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ENDOTHELIAL CELL GROWTH FACTOR

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(57) Claim

1. A polypeptide comprising recombinant platelet-derived endothelial cell growth factor.

22. A mammalian platelet-derived endothelial cell growth factor for use in preparing a therapeutic composition useful in treating wounds, atherosclerosis, angiogenesis, or thrombodytopenia.

12. An antibody to platelet-derived endothelial cell growth factor.

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COMPLETE SPECIFICATION

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COMPLETE SPECIFICATION FOR THE INVENTION ENTITLED:

Endothelial cell growth factor

The following statement is a full description of this invention, including the best method of performing it known to me/us:-

Platelet-derived endothelial cell growth factor (PD-ECGF) is a 45 kDa endothelial cell mitogen that has been purified to homogeneity from human platelets. It does not bind to heparin and does not stimulate the proliferation of fibroblasts, in contrast to other endothelial mitogens of the fibroblast growth factor (FGF) family. PD-ECGF appears to be the only endothelial cell growth factor in human platelets and recent data indicate that it has angiogenic activity in vitro, i.e., the ability to stimulate the formation of new blood vessels and chemotactic activity, in vitro. The present invention provides a homogeneous PD-ECGF in substantially greater yields than available in the past, the primary structure of PD-ECGF, antibodies against PD-ECGF, clones of its cDNA, and variants thereof. The invention also provides a therapeutic preparation of PD-ECGF.

Polypeptide growth factors play an important role in stimulating proliferation of target cells which are involved in both normal cellular processes and disease states. Some examples of processes that involve cell prolifration are, for instance, epidermal tissue replacement and immune system responses. Hemostasis, which is prevention of blood loss, is a continual process requiring that vascular tissue be constantly replaced. Growth of vascular endothelial cells has a central role in a variety of physiological and pathologicalprocesses, such as angiogenesis, woundhealing, atherosclerosis, and tumor

growth. In th case of vascular trauma, a compl x s ries of ev nts c mes into operation to maintain hemostasis. Initial response to trauma include activation of platelet plugging, blood coagulation, and eventually regrowth or repair of the damaged tissue. This final step must be activated at the proper time, place and in the proper tissue. One role for polypeptide growth factors is to mediate this response in both a temporal and tissue specific manner.

Peptide and polypeptide growth factors have been isolated from many sources. These include epidermal tissue, n utral tissue, platelets, placental tissue, and others. These peptide and protein factors are distinguished by a variety of properties including target cell specificity, h parin affinity or interaction, secretory properties, and physiochemical properties such as molecular weight, charge, h at stability, pH sensitivity, and susceptibility to reducing agents.

Endothelial cell growth is stimulated by a class of polypeptide growth factors known as fibroblast growth factors (FGFs). These mitogenic factors are characterized by their heparin-binding properties. Heparin is a powerful anticoagulant agent normally found in minute amounts in the circulatory system. The FGFs fall into two distinct protein classes, acidic and basic, and have been identified in neural and other tissues (Baird et al., 1986; Lobb et al., 1986; Thomas et al., 1986).

Recently, endothelial cell proliferation was shown to be stimulated by a novel polypeptide growth factor that was distinct from other known polypeptide growth factors derived from fresh human platelets (Miyazona et al., 1985a,b). This factor was originally called vascular ndothelial cell proliferation factor and was later renamed platelet-derived endothelial cell growth factor PD-ECGF.

Platelets are a rich source of growth factors f r a variety of hemop ietic and other tissu s. Platel t-d rived growth factors that have been identified and charact rized include platelet-derived growth factor (PDGF) which stimulates fibroblast and vascular smooth muscle cell growth but has no effect on endothelial cells; transforming growth factor- α (TGF-α) which is closely related to epidermal growth factor (EGF) and can stimulate growth of epidermal cells but not endothelial cells; transforming growth factor- β (TGF-β) which synergistically stimulates fibroblast growth in the presence of EGF and is furthermore a potent inhibitor of endothelial cell growth; a hepatocyte growth factor; and platelet-derived endothelial cell growth factor (PD-ECGF), the subject of this invention.

A growth-promoting activity was partially purified and characterized by Miyazono et al. (1987). The following characteristics were used to distinguish this activity from that of previously identified growth factors: Cultured porcine vascular endothelial cells were stimulated to incorporate ^{3}H thymidine into DNA in a dose-dependent manner upon treatment with a soluble lysate of fresh human platelets. The platelet lysate also promoted cellular proliferation by about 100% above a control culture treated with 1% fetal bovine serum. Fractionation of the lysate revealed that the activity appeared in the Mr 20,000 range on a Sephadex G-75 gel filtration column, stimulated ^{3}H thymidine incorporation into DNA of porcine vascular endothelial cells but not NRK fibroblasts, was probably distinct from PDGF which ran at Mr 30,000 on this column and stimulated fibroblasts but not endothelial cells, was more potent when prepared from fresh platelets rather than from outdated platelets, was heat-and acid-labile, resistant to the reducing agent dithiothreitol and was sensitive to

trypsin, guanidinium-HCl and urea, the latter two being denaturants. Based on these characteristics it was concluded that the growth-promoting activity was a polypeptide growth factor distinct from any that had previously been identified in human platelets.

Angiogenesis is the formation of new capillary blood vessels by sprouting from existing vessels. It is an important process in wound healing and tumor growth. Certain polypeptide factors have been identified which stimulate this process by virtue of their mitogenic or chemotactic activity for endothelial cells.

Mitogens are substances that stimulate proliferation of cells and are usually small molecules such as phorbal esters, polysaccharides, peptides, proteins or combinations thereof. Taxis is the directed migration of cells in response to an environmental cue. Chemotaxis is thus a response to chemicals in the environment. For eukaryotic cells, known chemotactic factors include histamine, amino acids, peptides, and proteins.

Polypeptide factors that elicit angiogenic responses include FGFs, TGF-α, TGF-β, tumor neurosis factor and angiogenin (Folkman et al., 1987a,b; Frater-Schroder et al., 1987; Leibovich et al., 1987). These factors exhibit multiple effects on a wide variety of cell types. Unlike these factors, human platelet-derived endothelial cell growth factor specifically stimulates endothelial cells.

Endothelial cell proliferation inside larger blood vessels is important for the regeneration of damaged endothelium and probably plays a role in prevention of atherosclerosis. Atherosclerosis is a condition in which lipid-rich lesions or plaques develop on blood vessels and can lead to numerous vascuous diseases. Alteration in the endothelial cell layer is thought to be an early event in

atherosclerosis. Platelets adhere to damaged endothelium cells and release mitogens to stimulate both endothelial and smooth muscle cell regeneration. PD-ECGF is among the mitogens that are released.

In disease associated with a reduction in platelet number, for example thrombocytopenia, PD-ECGF and other platelet mitogens and chemotactic factors may provide therapeutic benefit. Certain drugs can also induce platelet destruction or suppression. Thus, supplementing drug therapy with platelet mitogens may prevent untoward side effects.

The present source of PD-ECGF is fresh human platelets making purification of large amounts of this protein expensive and potentially risky given that human blood may contain infectious agents. Since PD-ECGF is heat labile, prior sterilization or pasteurization of blood will destroy its activity. To obtain quantities of PD-ECGF for further study and to provide therapeutic amounts, a more readily available source is desired. Cloning the PD-ECGF gene would allow the construction of expression vectors that produce large amounts of PD-ECGF.

The techniques of recombinant DNA technology are well established. While numerous manuals are available that outline the strategy and methodology for cloning genes, much of the work remains unpredictable; there is still no guarantee of success. Each cloning experiment can present its own special problems and pitfalls. A typical strategy for cloning eukaryotic genes is to isolate mRNA, to transcribe it into cDNA which is then inserted into a replicable vector, to transform a suitable host with the construct and to identify the desired clone by any number of means including functional activity or complementation, and screening with antibodies or oligonucleotides. A problem peculiar to some eukaryotic genes is finding a tissue in a

state of development or differentiation that is expressing the desired gene product, that is, containing the mRNA from the desired gene. The source of PD-ECGF is platelets which are enucleated cells and thus do not contain DNA or mRNA. Hence, to clone the gene for PD-ECGF a tissue or cultured cell line that produces PD-ECGF was needed to be found.

The present invention comprises homogeneous platelet-derived endothelial cell growth factor and an improved method for its purification to homogeneity from fresh human platelets. PD-ECGF is a 45 kDal endothelial cell mitogen that does not bind heparin and does not stimulate the proliferation of fibroblasts, in contrast to another class of endothelial cell mitogens which belong to the FGF family. PD-ECGF has angiogenic and chemotactic activity. With greater yields of PD-ECGF available, a partial amino acid sequence can be obtained from tryptic fragments as well as from V8-protease- and CNBR-derived fragments. The present invention provides proteolytic fragments of PD-ECGF and their use in obtaining the amino acid sequence of PD-ECGF and its variants. The PD-ECGF of the present invention provides variants of PD-ECGF.

The present invention relates to a polypeptide comprising recombinant platelet-derived endothelial cell growth factor.

The present invention further relates to an antibody to platelet-derived endothelial cell growth factor.

In addition, the present invention relates to a nucleic acid molecule comprising a recombinant DNA molecule or a cDNA molecule platelet-derived endothelial cell growth factor.

The present invention also relates to a mammalian platelet-derived endothelial cell growth factor for use in preparing a therapeutic composition useful in treating wounds, atherosclerosis, angiogenesis, or thrombodytopenia.

The present invention still further r lates to a process for the purification of homogeneous platelet-derived endothelial cell growth factor from a platelet lysate comprising subjecting the lysate to cation exchange chromatography, pooling and subjecting the resulting active fractions to anion exchange chromatography, pooling and subjecting the resulting active fractions to ammonium sulfate precipitation, pooling and subjecting the resulting active fractions to anion exchange chromatography, pooling and subjecting the resulting active fractions to high-performance hydroxylapatite column chromatography, pooling and subjecting the resulting active fractions to high-performance hydrophobic column chromatography, and recovering said homogeneous platelet-derived endothelial cell growth factor.

Fig. 1 illustrates chromatographic purification of human PD-ECGF on DEAE-Sepharose.

Fig. 2 illustrates chromatographic purification of human PD-ECGF on hydroxylapatite.

Fig. 3 illustrates chromatographic purification of human PD-ECGF on a Mono Q column.

Fig. 4 illustrates chromatographic purification of human PD-ECGF on a TSK-G4000 SW column.

Fig. 5 illustrates chromatographic purification of human PD-ECGF on a Superose 12 column. A) Column profile. B) SDS polyacrylamide gel of purified fractions of human PD-ECGF. C) SDS polyacrylamide gel of pure PD-ECGF in the presence or absence of a reducing agent.

Fig. 6 illustrates chromatographic purification of human PD-ECGF on a high-performance hydroxylapetite column. A) Column profile. B) Analysis of fractions by SDS gel electrophoresis and silver staining.

Fig. 7 illustrates chromatographic purification of human PD-ECGF on an alkyl Superose column. A) Column

profil . B) Analysis f fractions by SDS g l l ctr - phor sis and silver st ining. C) Analysis of th purifi d material by SDS electrophoresis and silver staining under nonreducing conditions.

Fig. 8 illustrates separation of tryptic peptide fragments of human PD-ECGF by narrow-bore reversed phase HPLC.

Fig. 9 shows the amino acid sequence of human PD-ECGF obtained by sequencing proteolytic fragments of PD-ECGF.

Fig. 10 illustrates an immunoblot analysis of a human placental extract.

Fig. 11 shows (A) restriction map, sequencing strategy and (B) nucleotide sequence of human placental DNA.

Fig. 12 illustrates (A) Southern blot analysis of human DNA and (B) Northern blot analysis of placental RNA.

Fig. 13 illustrates (A and B) biosynthesis of recombinant PD-ECGF in NIH3T3 cells and (C) immunoblot analysis of NIH3T3 cells expressing recombinant PD-ECGF.

Fig. 14 shows the chemotactic response to PD-ECGF by endothelial cells and smooth muscle cells.

Fig. 15 illustrates (A and B) induction of angiogenesis by PD-ECGF on the chick chorioallantoic membrane and (C) inhibition of induction of angiogenesis by antibodies that react with PD-ECGF.

Fig. 16 illustrates (A and B) induction of angiogenesis in mouse al-1 tumors transfected with cDNA clones expressing recombinant PD-ECGF.

Vascular endothelial cells make a functional monolayer between blood and underlying tissue. In a normal vessel wall, endothelial cells are known to exist in a

quiescent growth state. Proliferation of endothelial cells is a key component to a number of biological processes such as wound repair, thrombosis, atherosclerosis and tumor growth. Thus, factors that regulate endothelial cell proliferation play a key role in these conditions.

Factors with the ability to stimulate the formation of blood vessels are implicated as causative agents in angiogenesis of normal a malignant tissue, and this is reviewed by Folksman and Klagsburn (1987a). The most well-characterized factors in this category are FGFs which are a family of heparin-binding endothelial cell mitogens originally isolated from neural tissue but are also found in macrophages and other tissues. Another endothelial cell mitogen is PD-ECGF.

PD-ECGF growth-promoting activity can be assayed by determining the incorporation of ^{3}H-thymidine into the DNA of endothelial cells. Cells typically used in the assay are porcine aortic endothelial cells with bovine aortic endothelial cells, and human umbilical endothelial cells also being stimulated by PD-ECGF. Other established, cultured endothelial cell lines can be used in the assay with porcine aortic cells being preferred for routine assays.

Growth-promoting activity of PD-ECGF is also assayed by measuring cell proliferation. In this assay, fresh cells are plated in culture and the actual number of cells present is determined and compared to a control that has not received PD-ECGF. The cultured cell lines sutable for this assay are the sane as described in the ^{3}H-thymine incorporation assay.

When assayed on FGF cultures PD-ECGF does not stimulate incorporation of 3H thymidine into DNA nor does it induce cell proliferation.

PD-ECGF from platel ts, specially human platelets, has been purified in small quantities (Miyazono _t al., 1987). It has thus far been characterized as a 45 kDal polypeptide that is heat and acid labile, resistant to reducing agents such as dithiothreitol and others, and sensitive to proteolytic degradation, especially by trypsin and other serine proteases, and any of a number of well characterized proteases. The availability of larger amounts of pure PD-ECGF allows at least a partial amino acid sequence to be obtained in order to facilitate the cloning of its gene.

The purification of PD-ECGF is complicated by the fact that PD-ECGF occurs in low quantities in platelets and that its biological activity is both heat and acid labile. The process described by Miyazono et al. (1987) is a seven step process using both conventional chromatography and Fast Performance Liquid Chromatography (FPLC). The present invention contemplates an improved purification procedure that results in higher purity and substantially greater yields of PD-ECGF.

The present purification process begins with a side fraction of a platelet lysate in the purification of PDGF. This fraction is obtained by cation exchange chromatography on CM-Sephadex. The first step is anion exchange chromatography in batch on QAE-Sephadex. Next, the pooled fractions are concentrated by ammonium sulfate precipitation at about 42% of saturation. The third step is also anion xchange chromatography but on DEAE-Sepharose. The next three steps are all chromatographic involving adsorption chromatography on hydroxylapatite, anion exhange chromatography on Mono Q column attached to an FPLC system (Pharmacia), and gel filtration on a TSK-G4000 SW column.

The final step is hydrophobic interaction chromatography on a Superose 12 column.

The present invention is directed to a more rapid process having fewer steps for purification of PD-ECGF that results in about 1,250,000-fold purification at a yield of about 14%, preferably in five steps.

The process is the same as above up to and including the DEAE-Sepharose anion exchange chromatography. Other variations of the purification are also possible even up to this stage. For instance, the platelet lysate can be freshly prepared and need not be a side fraction in the purification of another factor such as PDGF. The chrmoatographic resins employed as described above are preferable for the purification process but other equivalent resins that are commercially available can be substituted with comparable results. Ammonium sulfate precipitation is also done at about 42% saturation; however, one skilled in the art can easily adjust this amount as necessary to precipitate the maximum amount of PD-ECGF activity.

The improvement in the purification process is a reduction in the number of steps following DEAE-Sepharose anion exchange chromatography and speeding the process by using more rapid chromatographic separation techniques that are provided by HPLC, FLPLC and the like. The present process provides homogeneous ECGF in higher yields and evidencing higher biological activity than previously available.

Specifically, the fractions obtained from the DEAE-Sepharose step are pooled and subjected to high performance affinity chromatography on an hydroxylapatite column. This is followed by a final purification step using high peformance hydrophobic interaction chromatography. The resin preferred for this step is alkyl-Superose but other

hydrophobic resin ar suitable. Such r sins are als commercially available. One of ordinary skill in the art can readily determine other suitable resins.

In another aspect of the present invention, antibodies are raised to PD-ECGF. Antibodies are useful in identifying a source of tissues or cells that are expressing PD-ECGF, since platelets are enucleate cells, and therefore, do not contain DNA for expression of the protein. The antibodies can be polyclonal or monoclonal either of which can be prepared by well known methods.

The antibodies of the present invention are raised against homogeneous endothelial cell growth factor, homogeneous PD-ECGF or recombinant PD-ECGF. The source of endothelial cell growth factor or PD-ECGF may be from mammals or humans and is purified to a level of homogenicity appropriate for raising antibodies. Polyclonal antibodies are prepared in rabbits, goats, sheep and rodents by injecting pure or partially purified PD-ECGF into the animal. After a prescribed series of injections, serum is obtained from the animal and tested for the presence of the desired antibody. The means of identifying a polyclonal antibody against PD-ECGF include immunoblotting, ELISA, Ouchterlony diffusion assay, radioimmuneassay, and other methods well krown in the art. (see for example, Johnstone et al., 1982). In the case of immunoblotting, pure PD-ECGF is run on an SDS acrylamide gel and then transferred to nitrocellolose by electrophoretic transfer in an appropriate buffer. After residual protein binding sites are blocked by any number of blocking agents including bovine serum albumin, Tween-20, non-fat dry milk, and gelatin or other blocking agents, the blot is treated with the antiserum to be tested. This is followed by detection of the bound antibody by a detection

means such as ^{125}I-Protein A with autoradiography or enzyme conjugated second antibodies which give a visible color or fluorescence upon treatment with their substrate.

Monoclonal antibodies against PD-ECGF are prepared by well-known methods. An outline of one method is to inject pure (or partially pure) PD-ECGF into a mouse, to remove its spleen after a response to the antigen has been mounted, to fuse the antibody-producing spleen cells to a myeloma line and to test the resulting hybridomas for production of an antibody that reacts with PD-ECGF by screening the hybridoma culture fluid. One method of screening is to use immunoblotting as described above. Alternatively, screening can be done by dot blotting or by immunofluorescence of a tissue or cell line that contains PD-ECGF.

The present invention provides a nucleic acid encoding the gene for endothelial cell growth factor. The nucleic acid may be a DNA or an RNA molecule, especially preferred is a complementary DNA molecule (cDNA). The cDNA molecule contains the nucleotide sequence encoding a mammalian or human endothelial cell growth factor. Alternatively, the cDNA contains the nucleotide sequence encoding mammalian or human PD-ECGF. The nucleotide sequence can be derived from the amino acid of human placental PD-ECGF or from the amino acid sequence of human platelet PD-ECGF. PD-ECGF differs from the placental source by ten amino acids and a single residue change of a leucine to a serine at Position 471.

The cloning of the PD-ECGF gene requires indentifying a source of mRNA from a cell line or tissue that expresses PD-ECGF because the major source of PD-ECGF in platelets which are cells that lack nuclei. Several hematopoetic cell lines were tested for the presence of PD-ECGF using either a polyclonal or monoclonal antibody and

immunoblotting of extracts of these tissues. It might be expected that hemotopoetic cells would also produce PD-ECGF, since they are related to platelets; however, none of the lines examined produced PD-ECGF. Human placental tissue was tested in the same manner and found to express PD-ECGF. Polyclonal antibodies would be expected to cross react with PD-ECGF from other mammalian sources. Thus, other tissues from humans and mammals can be readily tested by these methods to determine if they express PD-ECGF and provide sources of mRNA for cloning the PD-ECGF gene.

Purified poly (A) + mRNA is isolated by procedures that are well known. The method of Han (1987). is one such method but others can be found in standard laboratory manuals on recombinant DNA technology.

The selected mRNA is transcribed into a cDNA molecule using reverse transcriptase to make the primary strand, oligo (dG) tailing with terminal transferase and completing the double stranded molecule by priming with oligo (dC) primers. Other methods of preparing cDNAs for coloning can also be found in standard laboratory manuals on recombinant DNA technology.

The cDNA is inserted into a replicable vector which may include bacteriophage derivatives, especially preferred are lambda phage vectors which include but are not limited to λgt10 and λgt11.

The desired clone can be detected by a number of techniques. Antibodies can be used when a replicable expression vector such as λgt11 is used for the cloning. Radiolabelled oligonucleotides are useful in identifying a desired gene. The sequence for the oligonucleotide is deduced from the amino acid sequence of the PD-ECGF by the method of Lathe (1985) or by inspection of the genetic code for the given amino acid seuqence. Preferrably a region of

amino acid sequence is select d that maximizes th us of unique codons. Oligonucleotides can b synth sized by well-known methods on automated DNA synthesizers and can be any length from about 15 to about 100 nucleotides. The sequence of the oligonucleotide probes of the present invention are listed in the examples. Hybridization of the probes to clones carrying potential inserts is also well established and methods therefor can be found in standard laboratory manuals on recombinant DNA technology.

Clones that carry the PD-ECGF gene are sequenced by dideoxy chain termination by the method of Sanger or by the chemical method of Maxam and Gilbert.

The cDNA clones of PD-ECGF are useful for identifying homologous genes in mammals, determining the genomic-organization of the PD-ECGF gene in mammals, identifying PD-ECGF gene transcripts, cloning the genomic gene, especially human, and in constructing expression vectors to produce recombinant PD-ECGF from mammals, especially humans. Identifying homologous genes and determining the structure of the genomic gene are done by Southern hybridization of total genomic DNA. Cellular mRNA is analyzed by Northern blotting. Both Southern blotting and Northern blotting techniques are widely practiced and the methodology therefor is available in standard laboratory manuals on recombinant DNA technology. To clone the genomic PD-ECGF gene, the technology described in this invention is used except that the source of DNA is genomic DNA, and the hybridization probe is the cDNA clone rather than the oligonucleotides.

The deduced amino acid sequence of human placental PD-ECGF differs from human platelet PD-ECGF by one amino acid at position 471 where a leucine residue replaces a serine residue identified by direct amino acid sequencing. It also

contain d t n additional amino acids at th amin t rminus of the protein. It is unknown if this difference is the result of a processing event. Other variants of PD-ECGF may be cloned from other tissue sources such as bovine or porcine placental tissue and other tissues. These varients are useful for replacing human PD-ECGF in therapeutic uses that are described below.

Such vectors contain one or more selectable markers for maintainence of the plasmid and DNA sequence elements that are operably linked to a gene to control expression of that gene. These DNA sequence elements include promotors, enhancer elements, transcription termination signals and polyadenylation sites. The latter three are not always necessary and will depend on the replicable expression vector and the host system that is used to obtain expression. Promotors are DNA sequence elements that control gene expression. Prokaryotic promotors that are useful include the lac promotor, the trp promotor, the P_L and P_R promotors of lambda and the T7 polymerase promotor. Eukaryotic promotors are also useful in the invention and include promotors of viral origin and yeast promotors, especially the Molony Leukemia Virus LTR.

Expression of PD-ECGF is obtained by subcloning its gene into a replicable expression vector. Replicable expression vectors that are suitable for expression of PD-ECGF include bacterial and bacteriophage vectors that can transform such hosts as E. coli, B. subtilis and other microorganisms. Many of these vectors are based on pBR322 including Bluescript (commercially available from Stratagene) and are well known in the art. Bacteriophage vectors that are used in the invention include lambda and M13.

Other suitable vectors for PD-ECGF expression are derived from eukaryotic sources. Expression vectors that

function in y ast and cell culture are used to express PD-ECGF. These vectors includ yeast plasmids, retrovirus vectors, BPV vectors, bacullovirus vectors, and other viral vectors. Tissue culture cells that are used with eukaryotic replicable expression vectors include NIH3T3 cells, mouse L cells, COS-7 cells, HeLa cells and other extablished cultured cell lines, NIH 3T3 cells are preferred.

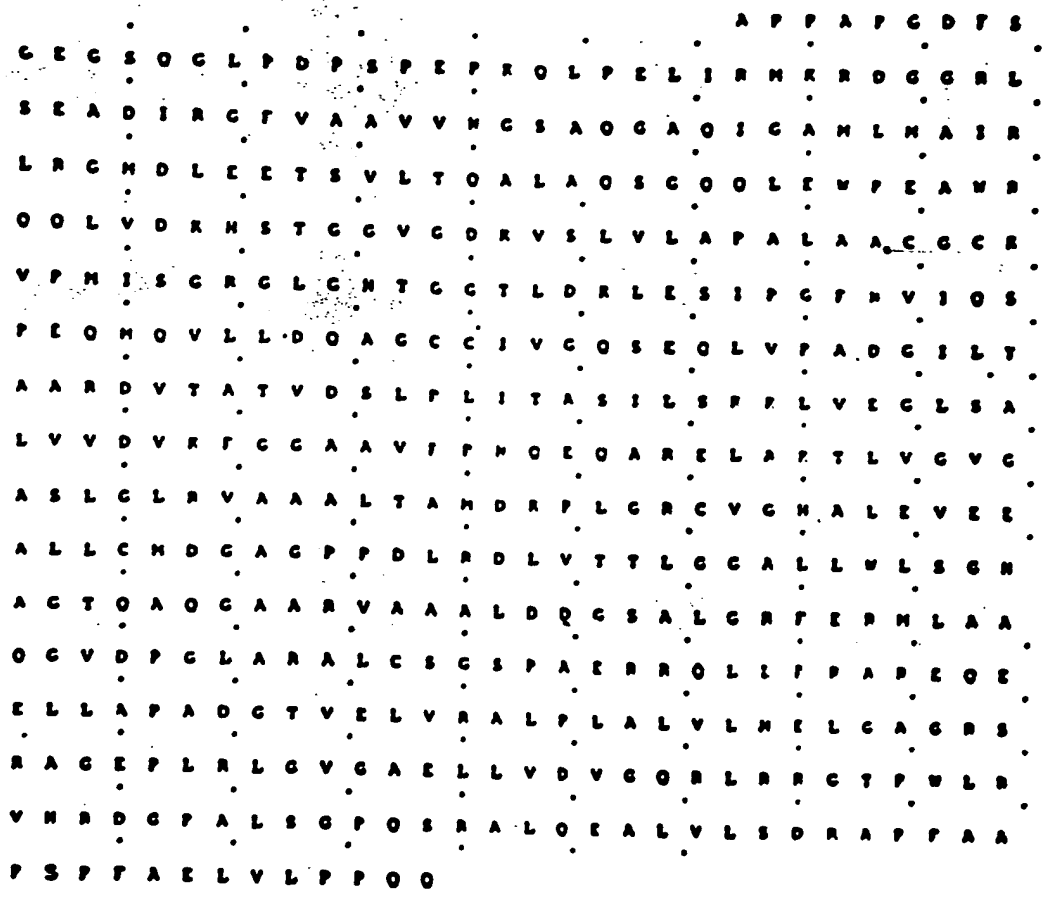
The replicable expression vectors of this invention are made by subcloning the cDNA insert from PL8 into the desired vector. In this invention, one replicable expression vector is plasmid pLJ, it contains the Molony leukemia virus LTR to drive the cDNA transcription and a neomycin gene as a selection marker. When the cDNA for PD-ECGF is subcloned into this vector, the plasmid pLPL8J is made. This vector is transformed to NIH3T3 cells by the $CaPO_4$ co-precipitation method. Analysis of a cell lysate and conditional medium of one cell transformed with pLPL85 revealed growth promoting activity for procine aortic endothelial cells in the cell lysate but not in the conditioned medoum. The activity is inhibited by PD-ECGF antibodies and upon immunoblotting with PD-ECGF antibodies reveals the presence of a recombinant PD-ECGF protein of 45 kDal.

Other replicable expression vectors are constructed and tested in a similar manner. One skilled in the art has available many choices of replicable expression vectors, compatible hosts and well known methods for making and using the vectors.

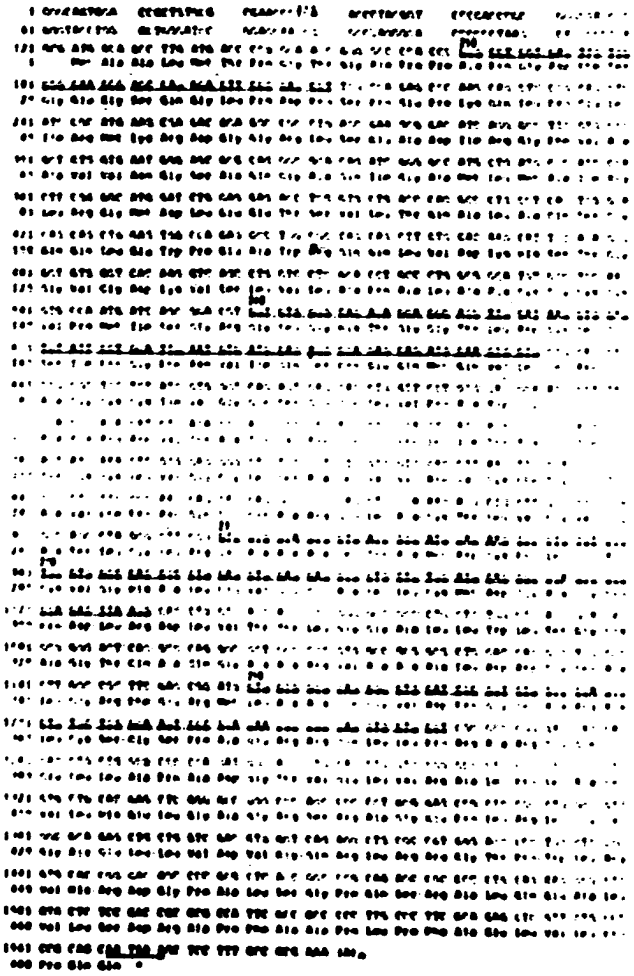
Transformant microorgansims and cultured cells are made by introducing a replicable expression vector into the system by transformation. Processes for transformation are well known in the art and include $CaCl_2$ treatment and electroporation of bacterial cells, $CaPO_4$ co-precipitation, protoplast fusion and electroporation for eukaryotic cells.

The detailed methods for these techniques can be found in standard laboratory manuals on recombinant DNA technology.

Homogeneous PD-ECGF was sequenced at the protein level and has the sequence:



Recombinant PD-ECGF was sequenced at the DNA level and the gene has a nucleotide sequence encoding PD-ECGP and flanking regions which are indicated below:



PD-ECGF stimulates chemotaxis and proliferation of endothelial cells *in vitro*, and induces angiogenesis *in vitro*. Angiogenesis, the formation of new capillary blood vessels by sprouting from preexisting vessels, is an important process in embryogenesis would healing, and organ

regen ration. In addition, aberrant angiogenesis occurs in sev ral pathological conditions, such as in tumor growth, in certain retinopathies, and in rheumatoid arthritis. Angiogenesis is a comples process that involves several steps, including migration and proliferation of endothelial cells (Folkman et al., 1987a). Certain polypeptide factors have been identified that stimulate this process, e.g. fibroblast growth factors, transforming growth factor-α and -β, tumour necrosis factor, and angiogenin (Folkman et al., 1987a; Frater-Schroder et al., 1987; Leibovich et al., 1987). These factors elicit the angiogenic response either directly by virtue or their mitogenic and/or chemotactic activity for endothelial cells or by as yet unknown indirect mechanisms. Unlike these factors which have multiple effects on a wide variety of cell types, a human platelet-derived endothelial cell growth factor (PD-ECGF) specifically stimulates the growth of endothelial cells in vitro (Miyazono et al., 1987), e.g. it does not stimulate growth of fibroblasts. PD-ECGF is a 45 kDa protein distinct from previously known growth factors.

Endothelial cell growth factor is useful in inducing angiogenesis, accelerating wound healing, preventing atherosclerosis, and treating thrombocytopenia by administering a therapeutic amount to a mammal. It is preferred that homogeneous or recombinant PD-ECGF be administered in therapeutic amounts to mammals including humans.

PD-ECGF also stimulates endothelial cell chemotaxis in vitro and induces angiogenesis in vitro in endothelial cells, especially the chick chorioallantoic membrane (CAM). Antibodies against the factor, which neutralize its mitogenic activity in vitro, also greatly inhibited the angiogenic

response. Thus, PD-ECGF is a novel angiogenic factor exhibiting target cell specificity towards endothelial cells.

To identify the chemotactic domain of PD-ECGF that induces endothelial cell chemotaxis in vitro, proteolytic fragments of PD-ECGF are tested for their ability to induce chemotactic responses of endothelial cells as described. Proteolytic fragments are prepared by enzymatic or chemical hydrolysis of PD-ECGF, especially by serine proteases-like trypsin, by V8 protease and by CNBR-induced cleavage. Fragments are purified by reverse phase HP66. The fragments are then tested in chemotaxis assays with mammalian endothelial cells, especially abovine and procine cells. The chemotaxis assay is the same that is used for testing the chemotactic activity of native or recombinant PD-ECGF.

The present invention shows that PD-ECGF is an angiogenic factor of a novel type. Stimulation of growth and chemotaxis of endothelial cells are in vitro properties that PD-ECGF has in common with other known angiogenesis factors (e.g. the FGFs). In contrast to these, however, PD-ECGF shows target cell specificity for endothelial cells in proliferation as well as chemotaxis assays. Thus, it can specifically and directly induce endothelial responses that are crucial for angiogenesis. PD-ECGF is the only endothelial mitogen present in a platelet lysate and may thus be an important factor involved in platelet mediated processes such as wound healing and the vascularization of thrombi. In addition, it may be of considerable importance as an intraluminal factor capable of regulating endothelial cell turnover if released from platelets. Endothelial cell proliferation inside larger vessels is important for the regeneration of damaged endothelium and thus probably important for the prevention of atherosclerosis. The availability of functionally active recombinant PD-ECGF

combined with a high yield purification method cDNA clones and specific antibodies will now make it possible to address questions related to the *in vitro* function of PD-ECGF, and to provide sufficient PD-ECGF for therapeutic use.

EXAMPLES

Cell Cultures and Media

Endothelial cells were collected from a fresh porcine aorta using collagenase digestion as described by Booyse *et al.* (1975). Cloning of the endothelial cells was performed by single cell platings as described originally by Puck *et al.* (1956). The endothelial cells were maintained in 25 cm^2 culture flasks in Ham's F-10 medium containing 10% fetal bovine serum (FBS) and antibiotics, and subcultured using 0.25% trypsin solution (Gibco) when the cells reached confluency. There was no evidence of transformation or loss of the endothelial monolayer under these conditions for more than six months. Normal rat kidney (NRK) fibroblasts were obtained by the method described by Duc-Nguyen *et al.* (1966) and subcultured in Dulbecco's modified Eagle medium containing 10% FBS.

Cell Number Determination

For growth experiments, endothelial cells were subcultured at a plating density of 20,000 cells/dish in a 35-mm tissue culture dish (Corning) using Ham's F-10 medium containing 10% FBS. The cells were incubated for 24 h to allow for attachment, and then the medium was changed to the test media. The cells were removed from the dishes by incubating for 15 min. in trypsin-EDTA and cell number was determined in duplicate. The cultures were refed with fresh test media on Day 4.

Preparation of Platel t Lysate

Blood was collected from normal volunt rs in citrate-phosphate-dextrose solution, and fresh platelet-rich plasma was obtained by centrifugation at 160 g for 20 min at 20°C. The upper four-fifths portion of platelet-rich plasma was centrifuged at 1700 g for 20 min. at 20°C, and the platelet pellet was suspended in 10 mM Tris HCl (pH 7.4)/150 mN NaCl/0.01 % polyethylene glycol (PEG). The platelets were washed twice with the same buffer in the centrifuge and sonicated for 1 min. By this method, erythocyte and leukocyte contamination in the last platelet suspension was less than 0.1%. Sonicated platelets were centrifuged at 48,000 g for 30 min. at 4°C and the supernatants were used as platelet lysate. About 2 ml of platelet lysate was obtained from 200 ml of whole blood. Further operations were performed at 4°C, unless otherwise specified.

Assay for Growth Promoting Activity

Growth promoting activity was determined by using porcine aortic endothelial cells as indicator cells, as previously has been described (Miyazono et al., 1985 a, b). Endothelial cells, cultured in Ham's F10 medium supplemented with 10% fetal bovine serum (Flow Laboratories) and antibiotics, were subcultured weekly at a ratio of 1 to 20. For mitogenic assay, the cells were trypsinized and re-plated sparsely (approximately 1 x 10^4 cells per well) with 500 ul of Ham's F-10 medium containing 0.5% fetal bovine serum and antibiotics in 24-well tissue culture plates (16 mm diameter, Costar). After 24 h of incubation, the test samples were added to the wells. 18 h later, [^{3}H]-thymidine (0.2 uCi/well; 6.7 Ci/mM, New England Nuclear) was added. After an additional 4 h, the cells were fixed with ice cold 5% (w/v)

trichloroacetic acid for 20 min. The resulting precipitat s wer washed extensively with water and solubiliz d with 200 ul of 1 M NaOH. After mixing at room temperature for 20 min, 200 ul of 1 M HCL was added to the wells. [^{3}H]-radioactivity was then determined in a liquid scintillation counter using 20 ml of Instagel (Packard) per sample.

Purification of Platelet-Derived Endothelial Cell Growth Factor from Human Platelets

Assay - Growth factor activity was monitored throughout the purification procedures using porcine aortic endothelial cells as target cells as described above.

Growth promoting activity on human foreskin fibroblasts was assayed by the method by Betsholtz and Westermark (1984).

General - During the purification, plastic utensils were used to decrease the loss of activity by adsorption to glass surfaces. All operations were performed at 4°C, unless otherwise specified. Protein concentration was determined by the dye fixation assay of Bradford (1976), unless otherwise specified.

Preparation of Platelet Lysate - For the purification of endothelial cell growth factor, a side fraction from the purification of platelet-derived growth factor (PDGF) from human platelets (Heldin et al., 1987) was used. The first step in the purification of PDGF from platelet pellets is a chromatography on CM-Sephadex. The cationic PDGF is adsorbed to this column. Since endothelial cell growth factor in human platelets is an anionic protein, it was not adsorbed to the CM-Sephadex column in the initial step of the purification of PDGF (Heldin et al., 1987). As expected, the

flow-through fraction was found to contain growth promoting activity for endothelial cells, and was used as starting material in the purification. About 15-liter of the non-adsorbed fraction was processed at a time; this was derived from about 900 l of fresh human blood. The non-adsorbed fraction was stored in 5 l containers at -20°C for up to 10 years until used.

QAE-Sephadex Chromatography - The non-adsorbed fraction from CM-Sephadex chromatography was thawed and processed to QAE-Sephadex chromatography. Dry QAE-Sephadex gel (0.7 g/l; A-50, Pharmacia) was added and mixed by shaking overnight. The gel was then allowed to sediment and, after the non-adsorbed fraction was discarded, it was poured into a column (60 x 5 cm, Pharmacia). The column was washed with 4 l of 75 mM NaCl in 10 mM phosphate, pH 7.4, and eluted with 2.5 l of 250 mM NaCl in 10 mM phosphate, pH 7.4.

The bulk of the activity bound to this column and luted between 75 and 250 mM NaCl at pH7.4. This resulted in a 25-fold purification estimated by the protein recovered, and an assumed recovery of 80% in this step. Since the growth promoting activity in the starting material was variable, maybe due to the presence of inhibitory substances (see below), the yield in this step could not be determined exactly.

Ammonium Sulfate Precipitation - Ammonium sulfate (247 g/l, 42 % of saturation) was added to the eluate of QAE-Sephadex chromatography. After equilibration for 2 h at 4°C, the sample was centrifuged at 2,075 x g for 15 min. The precipitate was collected by centrifugation and resuspended in 50 mM NaCl in 10 mM bis(2-hydroxylethyl) amino-tris-(hydroxymethyl)methane (Bis-Tris), pH 7.0.

At neutral pH, 42% saturation of ammonium sulfate precipitated about 90% of the activity, while only 9% of protein was coprecipitated. This procedure led to a 10-fold purification with abut 700 mg of protein remaining in 200 ml of volume.

DEAE-Sepharose Chromatography - The material obtained by ammonium sulfate precipitation (approximately 700 mg of protein in the volume of 200 ml) was treated with 5mM dithiothreitol at room temperature for 2 h. The sample was then dialyzed extensively against 50 mM NaCl in 10 mM Bis-Tris, pH 7.0, and applied to a column of DEAE-Sepharose CL-6B (40 ml; Pharmacia). The column was washed with 100 ml of 50 mM NaCl in 10 mM Bis-Tris buffer, pH 7.0, and eluted with a linear gradient (800 ml) of NaCl from 50 to 200 mM in 10 mM Bis-Tris, pH 7.0, at a flow rate of 120 ml/hr. Fractions of 20 ml were collected and analyzed for protein and growth promoting activity.

The endothelial cell growth promoting activity eluted at about 120-150 mM of NaCl at pH 7.0. This step led to a further 6-fold purification at a recovery of 50% (Fig. 1).

Hydroxylapatite Chromatography

The active fractions from DEAE-Sepharose chromatography (approximately 60 mg of protein) were combined and loaded directly onto an 8 ml column of hydroxylapatite (Clarkson Chemical Co.: Williamsport, PA) equilibrated with 50 mM NaCl, 0.6 mM phosphate, pH 7.4. The column was washed with 80 ml of the same buffer, eluted with 50 mM NaCl, 15 mM phosphate, pH 7.4, followed by 200 mM phosphate, pH 7.4. Fractions of 8 ml were assayed for protein and growth promoting activity.

Hydroxylapatite chromatography gave a further 4-fold increase in specific activity (Fig. 2). Almost all the protein bound to the gel, and the growth promoting activity eluted at 15 mM phosphate, whereas the bulk of protein eluted at a higher concentration of phosphate. The recovery of the activity was about 55% in this step, and about 8 mg of protein remained.

Final purification of the growth factor was achieved using ion exchange and high-resolving gel chromatography columns attached to a fast-performance liquid chromatography (FPLC) apparatus.

Mono Q Chromatography

The active fractions of the hydroxylapatite chromatography were pooled, centrifuged at 90,000 x g for 30 min at 4°C, and applied directly to a Mono Q column (HR 5/5, Pharmacia). This column and the following two (TSK-Gr000 SW and Superose 12), were attached to an FPLC-apparatus (Pharmacia) and operated at room temperature. The column was eluted at a flow rate of 1 ml/min with a gradient of 0-500 mM NaCl in 10 mM Bis-Tris, pH 7.0. Absorbance at 280 nm was monitored. Fractions of 1 ml were collected and tested for growth promoting activity.

A single peak of growth promoting activity was obtained at 120 mM NaCl at pH 7.0 (Fig. 3). This step led to a further 4-fold purification with 50% of recovery.

TSK-G4000 SW Gel Chromatography

The active fractions obtained from Mono Q column chromatography were pooled, lyophilized without prior dialysis and dissolved in 200 ul of water. The aliquot was then applied to a TSK-G4000 SW column (7.5 x 600 mm, LKB) with a precolumn (Ultropac column, 7.5 x 75 mm, LKB). The

column was equilibrated with 100 mM phosphate, pH 6.5, and eluted at a flow rate of 500 ul/min. The absorbance of the column effluent was monitored at 280 nm. Each 500 ul fraction was collected and tested for growth promoting activity.

The result was a single broad peak of activity eluting anomalously late, at a position corresponding to a M_r of less than 12,000 (Fig. 4). The recovery of the activity was 30% giving a 4-fold increase in specific activity.

Superose 12 Gel Chromatography

The active fractions obtained from TSK-G4000 SW chromatography were pooled, dialyzed using Spectrapor dialysis tubing (M_r cutoff, 3,500, Spectrum Medical Industries, Inc.) against 100 mM NaCl, 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), pH 7.0, and then lyophilized. The material was resuspended in 100 ul of water and run on a Superose 12 column (HR 10/30, Pharmacia) in 200 mM NaCl, 10 mM Hepes, pH 7.0. The column was eluted at a flow rate of 400 ul/min and monitored with the absorbance at 280 nm. Fractions of 200 ul were collected and assayed for growth promoting activity.

This procedure yielded a single sharp peak of bioactivity with an elution position between bovine serum, albumin and ovalbumin, well separated from the majority of protein (Fig. 5A). The increase in specific activity was 12-fold in this step at 30% recovery, giving 2 ug of pure material. The protein concentration in the purified fraction was estimated by comparing the intensity of the bands after sodium dodecyl sulfate (SDS)-gel electrophoresis and silver staining, with those of standards of bovine serum albumin.

The protein compositions of the fractions from the Superose 12 chromatography step were analyzed by SDS-gel

electrophoresis and silver staining (Fig. 5B). A homogenous protein with a M_r of 45,000 exactly coeluted with th growth promoting activity, suggesting that it represents the growth factor. A similar mobility was observed when the samples were analyzed under non-reducing conditions (Fig. 5C), suggesting that the endothelial cell growth factor from platelets is composed of a single polypeptide chain.

A summary of the purification procedure from 200 g of platelet protein is shown in Table 1. The overall increase in activity was about 1,000,000-fold at a recovery of 1%.

TABLE 1

Summary of the Purification of Platel t-D rived Endothelial Cell Growth Factor

purification step	protein (ug)	max stimulation (ng/mL)	purification (x-fold)	yield (%)
platelet lysate (flow-through from CM-Sephadex)	200 000 000	[a]	1	100
QAE-Sephadex	8 000 000	1 000 000	20[a]	80[a]
ammonium sulfate pptn	700 000	200 000	200	70
DEAE-Sepharose	60 000	17 000	1 200	36
hydroxyapatite	8 000	4 000	5 000	20
Mono Q	1 000	1 000	20 000	10
TSK-G4000 SW	70	240	83 000	3
Superose 12	2[b]	20	1 000 000	1

[a] The growth promoting activity in the platelet lysate was variable, maybe due to the presence of growth inhibitors (see, discussion). Therefore, the specific activity of the starting material could not be accurately determined. The purification in the QAE steps was estimated at 20-fold, based on the amount of protein recovered and an assumed recovery of 80% of activity in this step.

[b] Protein concentration was estimated by the relative intensities of bands in silver stained SDS-gels.

Improved Purification Protocol for PD-ECGF

The initial steps of the prufication wer performed as described above. As the next step, chrmoatography on an HPLC-grade hydroxylapatite column was used.

Chromatography on a High-Performance Hydroxylapatite Column. The material obtained from DEAE-Sepharose chromatography was filtered through a 0.22 um filter (Millipore) and loaded at room temperature onto a high-performance hydroxylapatite column (100 x 7.8 mm; BioRad) equipped with a guard column (50 x 4.0 mm; Bio-Rad). The column was preequilibrated with 1 mM phosphate buffer, pH 6.8, 50 mM NaCl, and 0.01 mM $CaCl_2$ and eluted at a flow-rate of 0.5 mL/min with a gradient of 1-100 mM phosphate, pH 6.8, 50 mM NaCl, and 0.01 mM $CaCl_2$. The column was then washed with 1 M phosphate buffer, pH 6.8, and 0.01 mM $CaCl_2$. Fractions of 1 mL were collected and tested for growth-promoting activity on porcine endothelial cells.

PD-ECGF bound to the gel in 1 mM phosphate buffer, pH 6.8, and was eluted with a linear gradient of phosphate (as indicated in Fig. 6A). The recovery of activity was about 32% in this step. Individual fractions from the chromatogram were analyzed by SDS gel electrophoresis and silver staining; the growth-promoting activity coeluted with two components of 46 and 44 kDa and some other components (Fig. 6B).

Chromatography on an Alkyl-Superose Column. The active fractions from the high-performance hydroxylapatite chromatography were pooled and mixed with an equal volume of 2.8 M ammonium sulfate (HPLC-grade, Bio-Rad) and 100 mM phosphate buffer, pH 6.8. The material was applied to an alkyl-Superose column (HR5/5, Pharmacia), preequilibrated with 1.4 M ammonium sulfate/100 mM phosphate buffer, pH 6.8, and eluted with a gradient of ammonium sulfate from 1.4 to

0 M in 100 mM phospat buffer, pH 6.8 as indicat d in Figure 7A. The flow-rate was 0.5 ml/min and the column was operat d at room temperature. The absorbanc of th column ffluent was monitored at 280 nm. Fractions of 500 ul were collected and assayed for grwoth-promoting activity.

Final purification was achieved by hydrophobic chr matography using FPLC. The pooled fractions from the high-performance hydroxylapatite chromatography were applied to an alkyl-Superose column, equilibrated with 1.4 M ammonium sulfate/100 mM phosphate buffer, pH 6.8, and eluted with a decreasing gradient of ammonium sulfate in 100 mM phosphate buffer, pH 6.8. A single peak of growth-promoting activity was obtained at about 0.8 M ammonium sulfate (Fig. 7A). The increase in specific activity was 50-fold in this step at a r covery of 82%. The protein compositions of individual fractions of the alkyl-Superose chromatogram were analyzed by SDS gel electrophoresis and silver staining (Fig. 7B); again, the active fractions contained the principal two components f 46 and 44 kDa, indicating that PD-ECGF was essentially pure. When the purified material was analyzed under n nreducing conditions, bands of the same sizes were observed (Fig. 7C). Furthermore, a very faint band of M_r 42,000 was reproducibly found. The proportion of these proteins in the silver-stained gels differed from preparation to preparation, but the 46- and 44-kDa components were always predominant.

A summary of the purification procedure starting from 300 g of platelet protein, corresponding to approximately 800-1000 L of human blood, is shown in Table 2. About 34 ug of pure PD-ECGF was obtained from each preparation. The material was purified 1,250,000-fold at an overall yield of about 14%.

TABLE 2

Summary of the Purification of PD-ECGP[a]

purification step	protein (ug)	max stimulation (ng/mL)	purification (x-fold)	yield (%)
platelet lysate (flow-through from CM-Sephadex)	300 000 000	[b]	1	[b]
QAE-Sephadex	12 000 000	1 000 000	20[b]	80
ammonium sulfate pptn	800 000	80 000	250	58
DEAE-Sepharose	80 000	10 000	2 000	53
high-performance hydroxyapatite	2 000	800	25 000	17
alkyl-Superose	24[c]	16	1 250 000	14

[a] The results represent mean values of four individual preparations.

[b] The growth-promoting activity in platelet lysate was variable, maybe due to the presence of growth inhibitors (Miyazono, et al., 1987). Therefore, the purification in the QAE-Sephadex chromatography was estimated at 20-fold on the basis of the amount of protein recovered and an assumed recovery of 80% of activity (Miyazono, et al., 1987).

[c] Protein concentration was determined by amino acid analysis.

SDS-Gel Electrophoresis

SDS-gel electrophoresis was performed according to the method described by Blobel and Dobberstein, 1975. Briefly, samples were heated (95°C, 3 min.) with or without 10 mM dithiothreitol, and applied to a gradient gel consisting of 10-18% polyacylamide. The gel was fixed with glutaraldehyde after electrophoresis and then silver stained (18). M_r markers (Pharmacia) containing phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin-inhibitor (20,100) and a-lactalbumin (14,400) were used.

Structural Analysis and Amino Acid Sequence of PD-ECGF

About 40 ug of pure PD-ECGF was reduced and pyridyl-ethylated, and then desalted using narrow-bore reversed phase HPLC. The material was then subjected to tryptic digestion followed by separation of the fragments on a narrow-bore reversed-phase HPLC column eluted in 0.1% trifluoroacetic acid with a gradient of acetonitrile.

For reduction and pyridyl-ethylation, PD-ECGF was first desalted on a C4 column and then dried in a Speedivac Concentrator and redissolved in 200 ul of 6 M guanidine-HCL, 0.25 M Tris-HCl, pH 8.5 and 2 mM EDTA, containing 100 ug of dithiothreitol. The solution was flushed with nitrogen for 20 s and left at room temperature for 3 h, at which time 2 ul of 4-vinyl-pyridine was added. After another three hours at room temperature, the sample was desalted by chromatography on the C4 column in the trifluoracetic acid/acetonitrile buffer system. The volatile solvent was removed as above and PD-ECGF was digested with TPCK-Trypsin (Sigma; enzyme to substrate ratio, 1/50 (w/w)) in 0.1 M ammonium bicarbonate, containing 2 M urea, for 4 h at 37°C. The tryptic fragments were immediately loaded onto a narrow bore reversed-phase

HPLC. The chromatographic equipment consisted of a dual pump LKB system, adapted for narrow bore chromatography and equipped with a variable wavelength detector. The column temperature was kept at 35°C, the flow rate was 100 ul/min and the effluents were monitored at 220 nm. Fractions were collected manually in polypropylene tubes.

The separation of tryptic fragments of PD-ECGF is illustrated in Figure 8A. Approximately 40 ug of trypsin-digested PD-ECGF was loaded onto a C4 reversed phase column (Brownlee Aquapore BU-300; 2.1 x 30 mm and eluted with a linear gradient of acetonitrile in 0.1% trifuoracetic acid.

Non-homogenous peptides were rechromatographed under different conditions (examples are given in Fig. 8B-E); Figures 8B, 8D and and 8E illustrate rechromatography of the material under peaks T19, T9, T5; T4, T21, T18; and T7, T13, respectively, of Figure 8A. The samples were diluted three-fold with 0.1% trifluoracetic acid and loaded onto a Brownlee Spheri-5 RP-18 column (2.1 x 30 mm). The column was eluted with 0.1% trifluoracetic acid and a linear gradient of acetonitrile indicated. Figure 8C shows rechromatography of the peptides under peak T12, T22 of Figure 8A. The column used was the same as in Figure 8A, but it was eluted with 0.15 M NaCl in Milli-Q water with a gradient of acetonitrile as indicated.

Single peptides were then subjected to N-terminal amino acid sequencing using a gas phase sequencer. (Applied Biosystems Protein Sequencer, model 470A, with an on line PTH-analyzer, model 120A). Sequence information from a total of tryptic peptides was obtained (Fig. 9). Additional sequence information was obtained by N-terminal sequencing of intact PD-ECGF, and by analysis of fragments obtained by digestion with CNBr of Staphylococcal V8 protease, using similar amounts of starting material.

In Fig. 9, tryptic fragments are indicated by (/...) and designations refer to peaks in Fig. 1. N-t rminal sequence (/---), Staphylococcus protease V8 fragments (/+++) and a CNBr fragment (/===) of PD-ECGF are also indicated, as well as cysteine residues (*), a potential N-glycosylation site (#), and a possible site of polymorphism (@). Internal repeats are overlined.

Preparation of Antibodies to PD-ECGF

To aid in cloning of cDNA for PD-ECGF, a specific polyclonal antiserum against PD-ECGF was raised and used in immunoblotting to localize a source of production of PD-ECGF.

The antiserum was prepared by diluting pure PD-ECGF to a concentration of 20 ug/ml in 10 mM phosphate buffer,, pH 7.4 with 150 mM NaCl. Then, ten micrograms of pure PD-ECGF was mixed with an equal volume of Freund's complete adjuvant and injected intramuscularly into a rabbit. The rabbit was boosted 2 weeks later with 10 ug of PD-ECGF in Freund's incomplete adjuvant. After that, the rabbit was boosted every 2-3 weeks with 5 ug of PD-ECGF in Freund's incomplete adjuvant. The immunoglobulin fraction was purified by applying 4 mL of immune serum to a 2-mL column of protein A-Sepharose (Pharmacia) equilibrated with 100 mM phosphate buffer, pH 7.4. The column was washed with the same buffer and then eluted with 50 mM citrate buffer, pH 3.0. The eluate from the column was rapidly neutralized with 1 M Tris-HCl, pH 7.4.

Since PD-ECGF occurs in platelets, several hematopoietic cell lines were tested, but none was found that synthesized PD-ECGF. A strong 45 kDa band was, however, found when an extract of a term human placenta was analyzed by immunoblotting. The human term placenta was minced and homogenized with 4 volumes of phosphate buffer saline (PBS).

To th homogenate was add d ammonium sulfat at 28% saturation; after centrifugation additional ammonium sulfate was added to the supernatant, to 42% saturation. The precipitate, recovered after centrifugation, was dissolved in PBS. Samples of 100 ng of PD-ECGF, purified from human platelets as described previously, and 375 ug of ammonium sulfate fractionated human placenta extract were subjected to SDS-gel electrophoresis in 10-18% gradient polyacrylamide gel under reducing conditions, and transferred to a nitrocellulose membrane in a buffer containing 20% ethanol, 150 mM glycine, and 20 mM Tris-HCl, pH 8.4, at 200 mA. The nitrocellulose membrane was incubated in 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10% bovine serum albumin to block non-specific binding, incubated in a 1:50 dilution of a specific rabbit PD-ECGF antiserum, washed twice with 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, and twice with 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.05% Triton X-100. The nitrocellulose membrane was then incubated with ^{125}I-labeled Staphylococcal protein A (5 x 10^5 cpm/ml), and washed as described above. Blots were subjected to autoradiography. PD-ECGF purified from platelets occurs as a doublet of about 45 kDa, probably due to proteolysis during preparation.

Figure 10 illustrates an immunoblot analysis of a human placenta extract (lane a) and PD-ECGF purified from human platelets (lane b) using the PD-ECGF polyclonal antiserum.

cDNA Cloning of PD-ECGF

A. Preparation of cDNA

A cDNA library was therefore constructed in λgt10 using poly(A)+RNA from human placenta.

Total RNA was isolated from a human placenta of 22-gestational-weeks by the method of Han, et al. (1987). Poly $(A)^+$RNA was purified by chromatography on an oligo(dT) column. A cDNA library was constructed in λgt10 following the method described by Watson et al. (1985) with some modifications. For synthesis of the first strand, cloned murine leukemia virus reverse transcriptase (Bethesda Research Laboratory) was used. Following the first strand synthesis, oligo(dG) was added to its 3' end by terminal transferase. Second strand synthesis was primed by addition of an external primer of oligo(dC), as well as internal RNA primers made by RNaseH.

B. Selection of Oligonucleotides and Screening

The information from amino acid sequencing of peptides was used to select and synthesize five unique ligonucleotides. Five unique oligonucleotides were deduced from the amino acid sequence by the method of Lathe (1985) and prepared by an Applied Biosystems DNA synthesizer 381A. The oligonucleotide sequences were as follows:

228,
5'TGTGTGGGCCATGCCCTGGAGGTGGAGGAGGCCCTGCTGTGCATGGATGGCGCT-GGCCCCCCTGACCTGCGG3'
231, 5'GTGGCTGCTGCCCTGACAGCCATGGACAAGCCCCTGGGCCGG3';
240,
5'GGCCTGGGCCACACAGGCGGCACCCTGGACAAGCTGGAGTCCATCCCTGGCTTCAATG-TGATCCAGTCCCCTGAGCAGATGCAGGTGCTG3';
258,
5'GCCCCCCCTGCCCCTGAGGACTTCTCTGGCGAGGGCTCCCAGGGCCTGCCTGACCCC3-';
259,
5'GTGGCTGCCCAGGGCGTGGACCCTGGCCTGGCCCGGGCCCTGTGCTCTGGCTCCCCTG-CTGAGCGGCGGCAGCTGCAGCC3'.

These oligonucleotide pr bes were lab led by end-tailing with a ^{32}p-dCTP and deoxynucleotide terminal transferase or were used as templates to synthesize radiolabeled complementary strands by oligonucleotide primers, a ^{32}P-dCTP and Klenow fragment.

The placenta library was screened with individual oligonucleotide probes. About three hundred thousand independent clones from the human placenta λgt10 cDNA library were plated and filter replicas were made using Hybond N nylon filters (Amersham). The filters were prehybridized in a solution of 20% formamide, 5 x SSC (SSC=150 mM NaCl, 15 mM sodium citrate), 50 mM sodium phosphate, pH 6.8, 1 mM sodium pyrophosphate, 5 x Denhardt's solution, 50 ug/ml denatured salmon sperm DNA and 100 uM ATP for 6 h at 42°C. Thereafter, labeled oligonucleotide probes were added to the solution and incubation prolonged for an additional 12-18 h. Filters were washed four times with 0.2 x SSC in 0.1% sodium dodecylsulfate (SDS) for 20 min each time. Washing temperatures were varied from 37°C to 42°C according to the oligonucleotide length. Filters were xposed to Kodak X-Omat AR films with Dupont Cronex intensifier for 12-48 h.

Three out of three hundred thousand clones were positive with two of the five probes, probes 228 and 240.

c. Subcloning and Sequencing

The three clones, λPL5, λPL7 and λPL8, were isolated and the cDNA inserts were subclones. The original clones λPL5, λPL7 and λPL8, have inserts of about 1.7, 1.0 and 1.8 kb, respectively (Bluescript, Strategene). Restriction mapping showed that the inserts were colinear with each other (Figure 4A). The restriction enzymes used were: B, BamHI; K, KpnI; S, SalI; and Sm, SmaI.

The inserts were also cloned into M13mp18 for sequencing. The nucleotide sequencing strategy for the λPL8 insert, the longest insert, is also shown in Fig. 11A. Double stranded forms of M13 were subjected to sequential deletion using exonucleases III and VII following the method of Yanisch-Perron, et al. (1985). Single-stranded DNAs were purified from each deletants and nucleotide sequences were determined by the dideoxy-termination method (1970). Sequences which were difficult to determine by this procedure were examined by the Maxam-Gilbert method (1980).

Nucleotide sequence of the λPL8 insert and deduced amino acid sequence of PD-ECGF is shown in Figure 11B. The putative initiation codon is at nucleotides 124-126. An in-frame stop codon (nucleotides 28-30) upstream of the initiation codon is underlined, and a stop codon at nucleotides 1570-1572 is marked with an asterisk. The polyadenylation signal (nucleotides 1566-1573) is underlined with an arrow. Regions corresponding to the prepared oligonucleotide probes, 226, 231, 240, 258 and 259, are underlined with broken lines.

Sequence of PD-ECGF

Nucleotide sequencing of the λPL8 insert revealed a short GC-rich 5' untranslated region, an open reading frame predicting the translation of a 482 amino acid long protein (M_r 49,971), and a short 3' untranslated sequence sequence containing a poly $(A)^+$ tail (Fig. 11B). The translation is probably initiated at the ATG at nucleotides 124-126, since the surrounding nucleotide sequence follows the rules for translation initiation, whereas ATG at nucleotides 136-138 does not. Furthermore, there is no other ATGs between an in frame stop codon at nucleotides 28-30 and the nucleotides coding for the N-terminus of intact PD-ECGF (Fig. 9). A stop

codon (TAA) is present at nucleotides 1570-1572. This stop codon is part of the polyadenylation signal (nucleotides 1568-1573). Fourteen nucleotides down-stream of this signal, a long stretch of poly(A)$^+$ was found. A similar overlap of the stop codon and the polyadenylation signal has been reported for human choriogonadotropin B-chain.

Out of the 482 amino acids of PD-ECGF deduced from the cDNA clone, 389 were identified by amino acid sequencing (Fig. 9). The N-terminal sequence of PD-ECGF starts 10 amino acids downstream of the proposed translation initiation site, indicating that the molecule undergoes a limited proteolytic processing after synthesis. The C-terminal amino acids predicted from the cDNA sequence, except the last four, were identified by amino acid sequencing. It is not known whether PD-ECGF undergo sproteolytic processing in the C-terminus, if so, a maximum of four amino acids are removed. The M_r of the mature protein would thus be 48,600-49,000, in reasonable agreement with the estimate of 45,000 obtained from SDS-gel electrophoresis of pure PD-ECGF.

The predicted amino acid sequence from the cDNA clone matched perfectly the previously obtained amino acid sequence in all positions, except in position 471, where the nucleotide sequence predicts a leucine residue, whereas a serine residue was found in both of two different peptides obtained from this region (Fig. 9). It is possible that polymorphism occurs at this position.

Matching the sequence of PD-ECGF with those of the PIR, EMBL and Genbank databases (releases 15, 14 and 56, respectively) revealed no striking homologies to other proteins. Short internal repeats in the sequence were noted (overlined in Fig. 2). One important feature of the sequence is the lack of a hydrophobic signal sequence suggesting that PD-ECGF is not a classical secretory protein. The sequence

contains only one tyrosine residue, which probably is not exposed on the surface of the molecule, since attempts to radiolabel PD-ECGF with ^{125}I using the chloramine T method, resulted in a very low incorporation of radioactivity. There is one potential N-glycosylation site, Asn-63 (Fig. 9). The PTH-amino acid yield of the corresponding tryptic peptide decreased dramatically, just before this residue (Fig. 9). This could be due to the formation of a cyclic compound of the asparagine residue and the following glycine residue, which can occur only if the aspargine residue is nonglycosylated. This, in combination with the M_r of PD-ECGF as determined by SDS-gel electrophoresis, compared with the M_r predicted from the cDNA clone, suggest that Asn-63 is not glycosylated. There are a total of 7 cysteine residues, indicating that the molecule contains at least one free SH group.

Southern and Northern Blotting

To obtain information about the genomic structure of the human PD-ECGF gene, Southern blotting analysis of human DNA was performed using the inert of pPL8 as a probe. High-molecular-weight human genomic DNA was prepared from normal human leukocytes. Samples were digested by restriction enzymes, subjected to electrophoresis in agar and blotted to nitrocellulose membranes (Schleicher and Schull). As shown in Fig. 12A, ten ug of human placenta DNA was cut with HindIII (lane 1) or XbaI (lane 2), and then subjected to electrophoresis in agar and hydridized by a PD-ECGF cDNA probe from the pPL8 insert. Single bands of 19 kb and 9.2 kb was observed after digestions with HindIII and XbaI, respectively. This suggests that only one copy of the PD-ECGF gene is present in the human genome. Indeed, analysis of human genomic PD-ECGF clones supports this conclusion.

Human placenta poly(A)$^+$RNA was examined by Northern blotting using the pPL5 insert as a probe. Poly(A)$^+$RNA was purified from 22-weeks-gestational human placenta as described above. One ug of RNA was electrophoresed in a 0.9% formalin denaturing gel and blotted to Hybond N nylon filter (Amersham). Hybridization was preformed in a solution of 50% formamide, 0.65 M NaCl, 0.1 M sodium Pipes, pH 6.8, 10% dextran sulfate, 5x Denhardt's solution, 0.1% SDS, 5 mM EDTA and 100 ug/ml salmon sperm DNA at 42° for 18 h, and then washed four times with 2 x SSC, 0.2% sodium phosphate, 0.1% SDS for 20 min. periods at 50°C.

A single transcript of about 1.8 kb was observed (Fig. 12B). Since the longest cDNA clone found, PL8, has an insert of about 1.8 kb, it most likely represents a full-length copy of the PD-ECGF transcript.

Expression of PD-ECGF cDNA

In order to verify the authenticity of the cDNA clone and to provide a replicable expression vector carrying the PD-ECGF gene, it was expressed in NIH3T3 cells. The pPL8 insert was subcloned into the pLJ expression vector to give pLPL8J. This vector has Moloney leukemia virus LTR as a promoter to drive the cDNA transcription, as well as a neomycin resistance gene as a selection marker Miwnica t al., 1986. The pLPL8J and pLJ constructs were introduced in the NIH3T3 cells by the calcium phosphate co-precipitation method, and cell lines were selected by neomycin.

NIH3T3 cells transfected with pLJ or with pLPL8J were grown to confluence in 10 cm cell culture dishes in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% calf serum and antibiotices. The cells were then cultured for 24 h in serum-free consitions in DMEM supplemented with 1% bovine serum albumin, 7.8 ug/ml

cholesterol, 5.5 ug/ml oleic acid, 8 ug/ml L-a-phosphatidyl-
ch line and 0.2 mg/ml transferin. After collecting the conditioned media, the cells were washed twice in PBS and then scraped into 1 ml of PBS. Following careful resuspension, a cell lysate was prepared by disrupting the cells by three cycles of freezing and thawing, followed by centrifugation at 25,000 g for 30 min. and collection of the supernatants. For the preparation of cell lysates, the cells were washed and resuspended in PBS containing 1 mM phenylmethylsulfonyl fluoride (Sigma), 150 KIU aprotinin (Sigma) and 10 mM EDTA. Growth-promoting activity was measured by the incorporation of ^{3}H-thymidine into porcine aortic endothelial cells in the absence or presence of 2 ug/ml of PD-ECGF antibody purified by protein A-Sepharose. Immunoblotting was performed as described.

Analysis of cell lysate and conditioned medium of one cell line transfected with pLPL8J, revealed growth promoting activity for porcine aortic endothelial cells in the cell lysate, but not in the conditioned medium (Fig. 13A,B). The activity was completely neutralized by a specific rabbit antiserum against PD-ECGF, indicating that the activity was due to the synthesis of functionally active PD-ECGF by the cell in (Fig. 13A). The NIH3T3 cell line transfected with pLJ did not contain any growth promoting activity for porcine aortic endothelial cells. The cell lysate of the cell line transfected with pLPL8J was furthermore found to contain a 45 kDa component in immunoblotting experiments using the antiserum against PD-ECGF (Fig. 13C). The size of the product was similar to that of human PD-ECGF purified from platelets (Fig. 13C). These data show that the purified, sequenced and cloned PD-ECGF molecule is responsible for the observed biological

ffect on porcine endothelial c lls. Thes data additionally showed that recombinant PD-ECGF can b expr ssed in cultur d cells.

Chemotactic Responses to PD-ECGF

The effect of PD-ECGF on the migration of bovine aortic endothelial cells (BAEC) and smooth muscle cells (SMC) was determined.

Chemotaxis assays of BAEC and SMC were performed in 48-well micro chambers. Gelatin and fibronectin coating of the Nucleopore filters was necessary for BAEC adhesion. Cells (18,000 BAEC, 25,000 SMC per well) were added to the upper wells. Serum-free Dulbecco's modified Eagle's medium (DMEM) was used for SMC migration, DMEM was supplemented with 1% FBS for BAEC migration. The number of cells which had migrated during a 5 h incubation period to the lower surface of the filter were counted in three high power fields. All experiments were done in triplicate. In Fig. 14A, the Dose dependent migration of BAEC is indicated by closed circles and on left ordinate and similarly for SMC by open circles and on right ordinate. Controls are shown as a histogram I) BAEC medium only, II) BAEC medium plus 10ng/ml basic FGF, III) SMC medium only, IV) SMC medium plus 5% FCS.

Fig. 14a shows the dose-dependent effect of PD-ECGF on the migration of bovine aortic endothelial cells (BAECs), which at saturation (5-10 ng/ml) is comparable to the potency of basic FGF as a chemotactic factor. Half-maximal stimulation occurred at a concentration of about 1 ng/ml. Consistent with its mitogenic target cell specificity for endothelial cells PD-ECGF did not induce smooth muscle cell migration under assay conditions. Controls using serum and platelet-derived growth factor induced extensive smooth muscle cell migration in the same experiment.

Checkerboard analysis revealed that BAEC migration induced by PD-ECGF is due to directed cell migration (chemotaxis) and not random migration (chemokinesis). The data are shown in Fig. 14B and represent percent cell number per field. Standard deviations were below 15%. The values on the diagonal indicate chemokinetic migration and the values below the diagonal indicate chemotaxis.

Finally, antibodies which neutralize the mitogenic activity of PD-ECGF in vitro also neutralized the chemotactic activity (Fig. 14C) indicating that PD-ECGF is responsible for this activity.

The inhibition of BAEC migration using PD-ECGF antibodies was performed as described above and the examples shown in Fig. 14C are 1) Control, 2) 5ng/ml pure PD-ECGF, and 3) 5ng/ml pure PD-ECGF plus 300 ng/ml anti PD-ECGF antibodies.

Angiogenic Properties of PD-ECGF

The effect of PD-ECGF in vivo was tested on the developing vascular system of the chick chorioallantoic membrane (CAM). Test substances were incorporated into methylcellulose disks (10ul) and transplanted onto a 9 day old CAM as described by Risan, et al. (1986). CAMs were analyzed daily for two days. Disks are visible by their light reflections. Fig. 15 shows the induction of Angiogenesis on the chick chorioallantoic membrane by a) partially purified PD-ECGF (purified through the hydroxylapatite step, 1% pure, 1.2 mg of protein/ml; 5 ul per methylcellulose disk), b) 50 ng pure PD-ECGF, and c) partially purified PD-ECGF (as in a) incubated for 20 min with anti-PD-ECGF antiserum (2.5 ul of each solution) (magnification x 10).

Partially purified PD-ECGF consistently induced a str ng angiogenic response (Fig 15A). Furth rmor , pur PD-ECGF at a dose of 50 ng induced angiogen sis in the CAM (Fig. 15B). These results are summarized in Table 3. Antibodies against PD-ECGF greatly inhibited PD-ECGF induced angiogenesis in this assay (Fig. 15C). Thus, the angiogenic r sponse is unlikely to be due to inflammation or other factors than PD-ECGF present in the preparation.

TABLE 3

Angiogenic Properties of PD-ECGF on the CAM

TRANSPLANT	ANGIOGENIC REACTION		
	++	+	-
Partially purified PD-ECGF (2.5-5 ul/disk)	54	4	7
Partially purified PD-ECGF (2.5 ul/disk) plus anti-PD-ECGF serum (2.5 ul/disk)	7	2	13
Pure PD-ECGF (50 ng/disk)	11	3	1

Number of samples is indicated in each column.

++: Strong angiogenic reaction

+: Less pronounced angiogenic reaction, in some cases only few vessels converging to the transplant

-: Unaffected vascular pattern

To investigat the *in vivo* ffects of PD-ECGF, pLPL8J and pLJ without insert were transfected by electroporation into a1-1 cells. a1-1 is a NIH 3T3 cell transformed by human activated H-ras and is tumorigenic in nude mice. After transfected a1-1 cells were selected by G418 resistance, they were injected into nude mice subcutaneously. In two weeks, tumors grew to be about 2 cm in diameter and they were harvested and fixed in formaldehyde. Histological examination (Figure 16) revealed that tumors developed from a1-1 transfected with pLPL8J have marked blood vessels in contrast with the few vessels in tumors from a1-1 transfected with pLJ without cDNA inserts. This observation indicates that human PD-ECGF is angiogenic in mice.

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CLAIMS

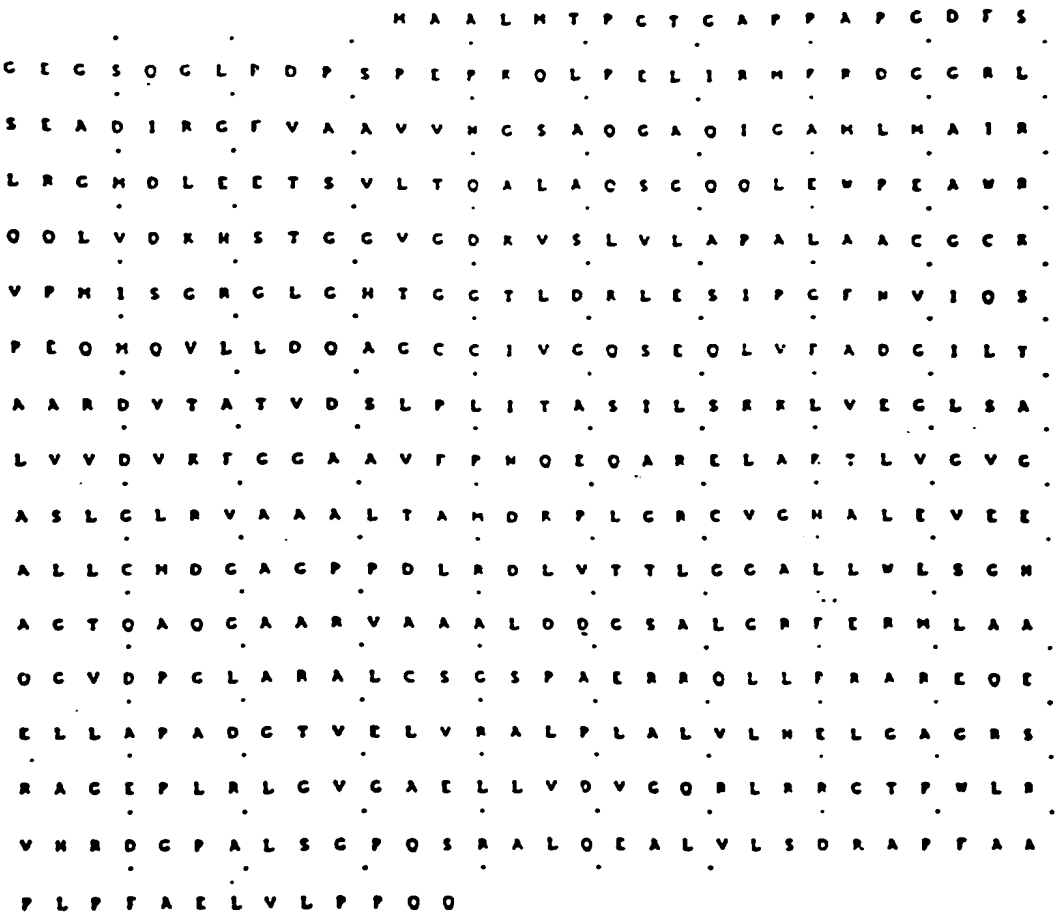
THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS.

1. A polypeptide comprising recombinant platelet-derived endothelial cell growth factor.

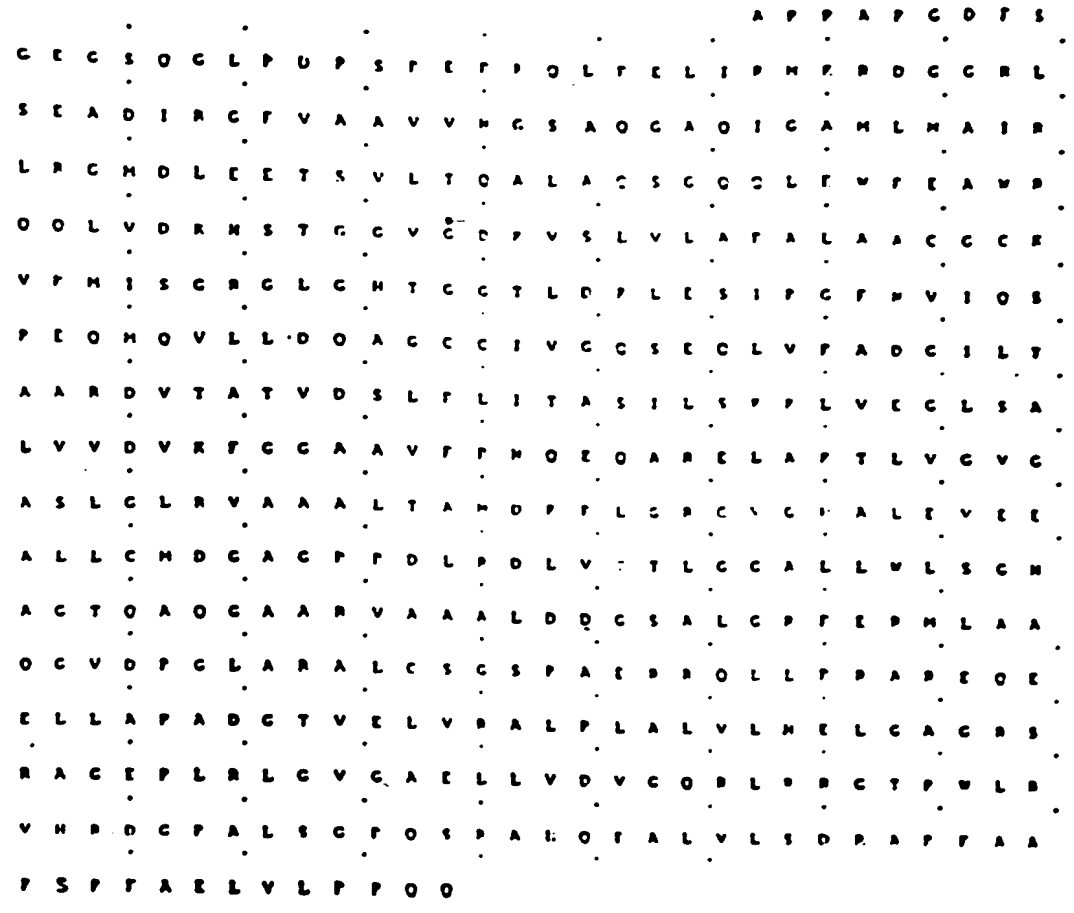
2. The polypeptide of Claim 1 wherein said platelet-derived endothelial cell growth factor is des-MAALMTPGTG endothelial cell growth factor.

3. The polypeptide of Claim 1 wherein said endothelial cell growth factor is mammalian or human.

4. The recombinant polypeptide of Claim 1 or 3 comprising the amino acid sequence:



5. The recombinant polypeptide of Claim 2 comprising the amino acid sequence:



6. The polypeptide of any one of Claims 1-5 wherein the leucine at position 471 of said sequence is replaced with serine.

7. A polypeptide comprising fragment of the platelet-derived endothelial cell growth factor of any one of Claims 1-6.

8. The polypeptide of Claim 7 wherein said fragment is obtained by enzymatic digestion.

9. The polypeptide of Claim 8 wherein said enzymatic digestion is effected by trypsin, chymotrypsin, pepsin, subtilisin, V8 protease or mixtures thereof.

10. The polypeptide of Claim 7 obtained by chemical or spontaneous cleavage of said factor.

11. The polypeptide of Claims 9-10 wherein said endothelial cell growth factor is recombinant or naturally-occurring.

12. An antibody to platelet-derived endothelial cell growth factor.

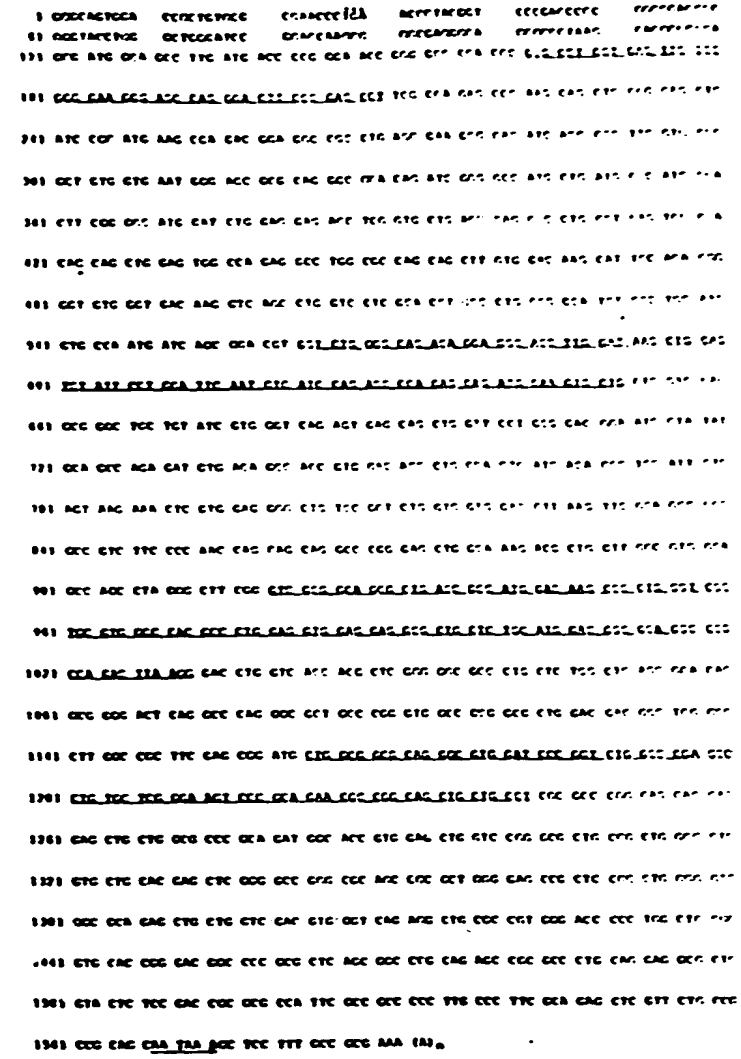
13. The antibody of Claim 12 wherein said antibody is polyclonal or monoclonal.

14. The antibody of Claim 12 or 13 wherein said factor is recombinant.

15. A nucleic acid molecule comprising a recombinant DNA molecule or a cDNA molecule platelet-derived endothelial cell growth factor.

16. A nucleic acid molecule comprising a recombinant DNA molecule or a cDNA molecule having a nucleotide sequence encoding an amino acid sequence of the endothelial cell growth factor of any one of Claims 1-6.

17. The nucleic acid molecule of Claim 15 comprising the nucleotide sequence:



18. A replicable expression vector comprising a nucleic acid molecule according to any one of Claims 15-17 which is operably linked with a nucleic acid sequence capable of effecting expression of said growth factor.

19. A transformant microorganism or cell culture transformed with the replicable expression vector of Claim 18 which is capable in said transformant microorganism or cell culture of expressing said endothelial cell growth factor.

20. The cell culture according to Claim 19 comprising transformed NIH 3T3 cells.

21. A microorganism according to Claim 19 comprising a transformed E. coli strain.

22. A mammalian platelet-derived endothelial cell growth factor for use in preparing a therapeutic composition useful in treating wounds, atherosclerosis, angiogenesis, or thrombodytopenia.

23. The growth factor of Claim 22 wherein said growth factor is recombinant.

24. A process for the purification of homogeneous platelet-derived endothelial cell growth factor from a platelet lysate comprising subjecting the lysate to cation exchange chromatography, pooling and subjecting the resulting active fractions to anion exchange chromatography, pooling and subjecting the resulting active fractions to ammonium sulfate precipitation, pooling and subjecting the resulting active fractions to anion exchange chromatography, pooling and subjecting the resulting active fractions to high-performance hydroxylapatite column chromatography, pooling and subjecting the resulting active fractions to high-performance hydrophobic column chromatography, and recovering said homogeneous platelet-derived endothelial cell growth factor.

25. The steps, features, compositions and compounds disclosed herein or referred to or indicated in the specification and/or claims of this application, individually or collectively, and any and all combinations of any two or more of said steps or features.

DATED this EIGHTEENTH day of DECEMBER 1989

Research Corporation Technologies, Inc.

by DAVIES & COLLISON
Patent Attorneys for the applicant(s)

DRAWINGS

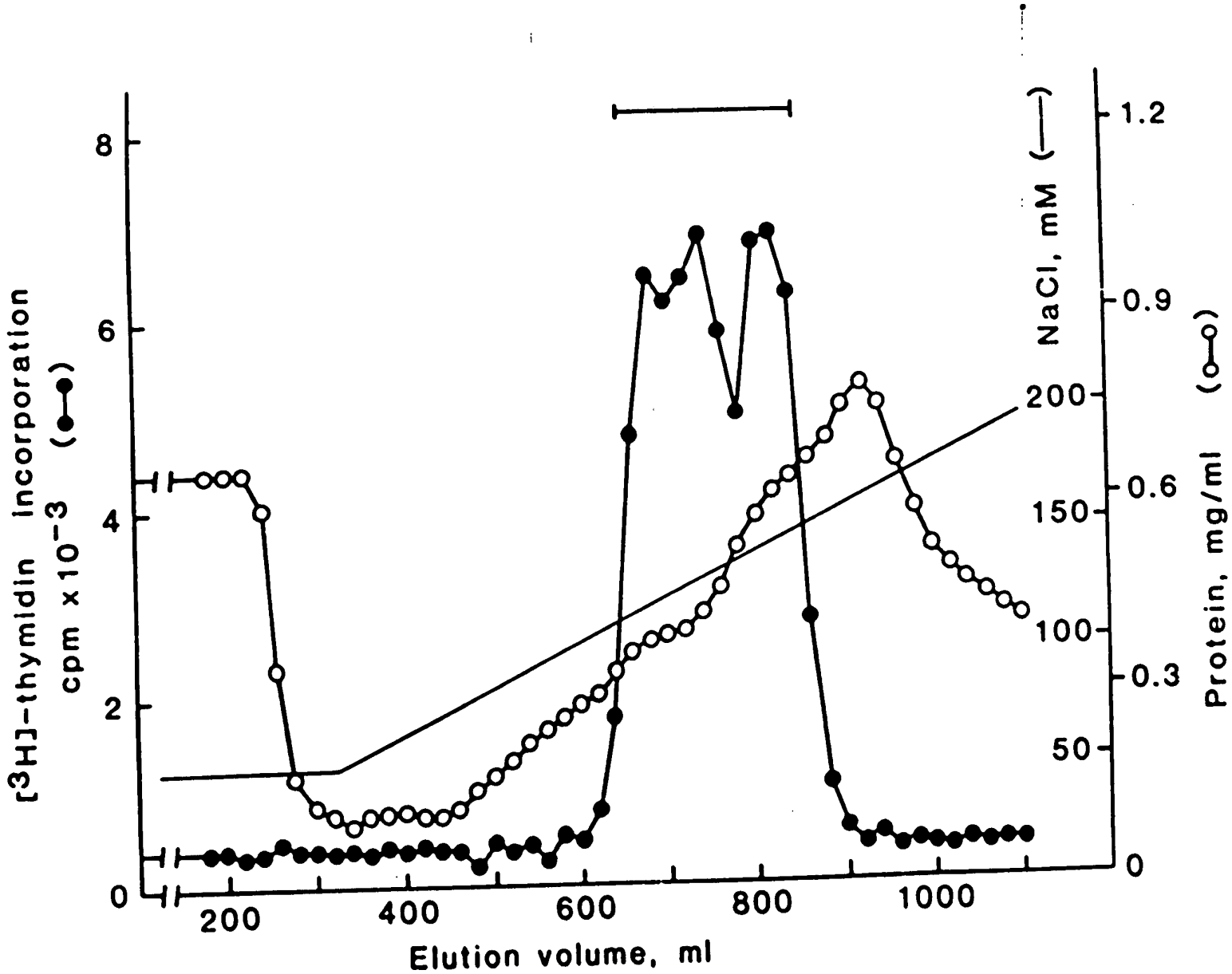

[3H]-thymidin incorporation
cpm x10-3 (●—●)
NaCl, mM (—)
Protein, mg/ml (o—o)
Elution volume, ml
0
2
4
6
8
200
400
600
800
1000
50
100
150
200
0
0.3
0.6
0.9
1.2

FIG.1

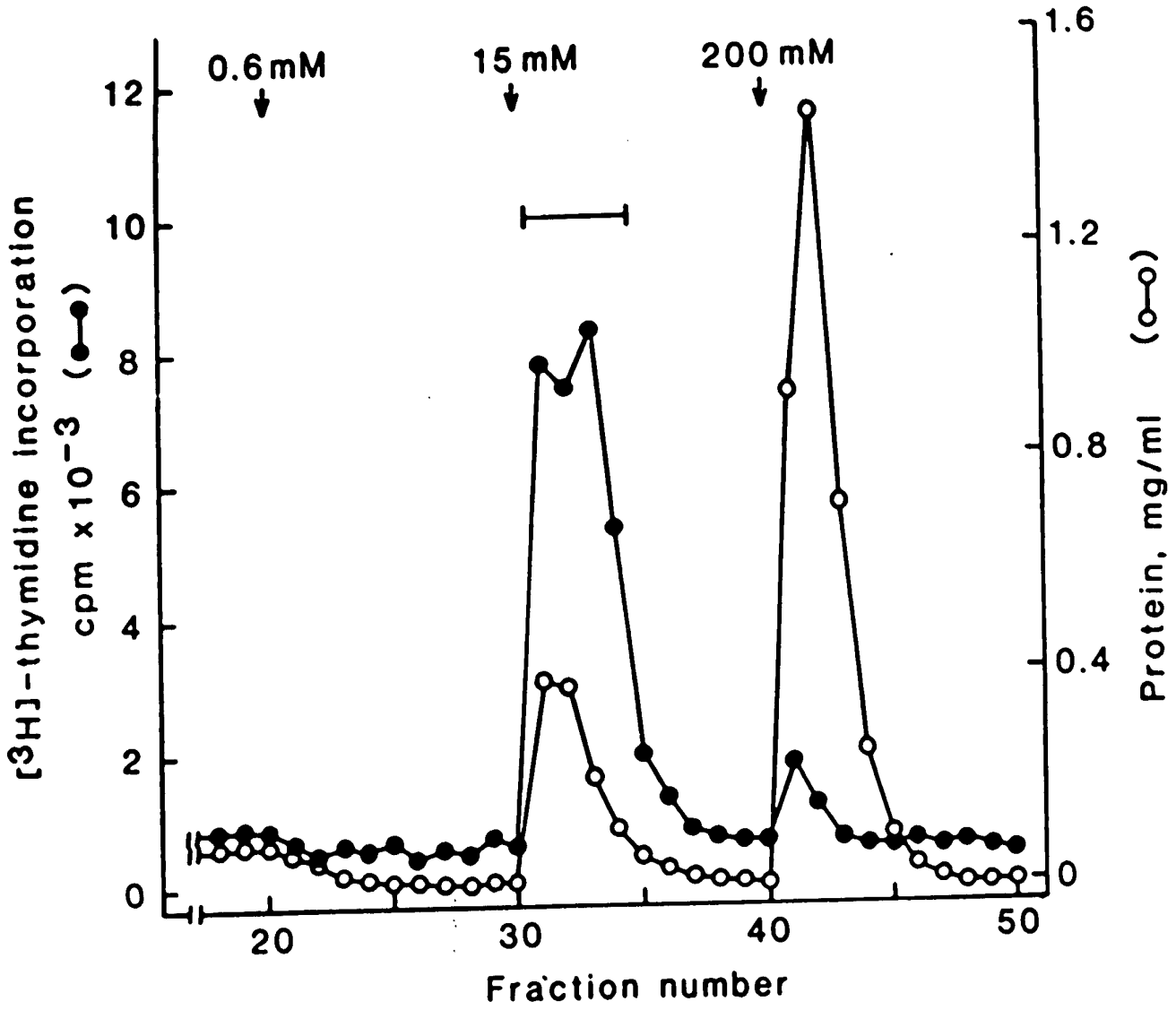

0.6mM
15mM
200mM
12
10
8
6
4
2
0
[3H]-thymidine incorporation
cpm x10-3 (●—●)
1.6
1.2
0.8
0.4
0
Protein, mg/ml (○—○)
20
30
40
50
Fraction number

FIG.2

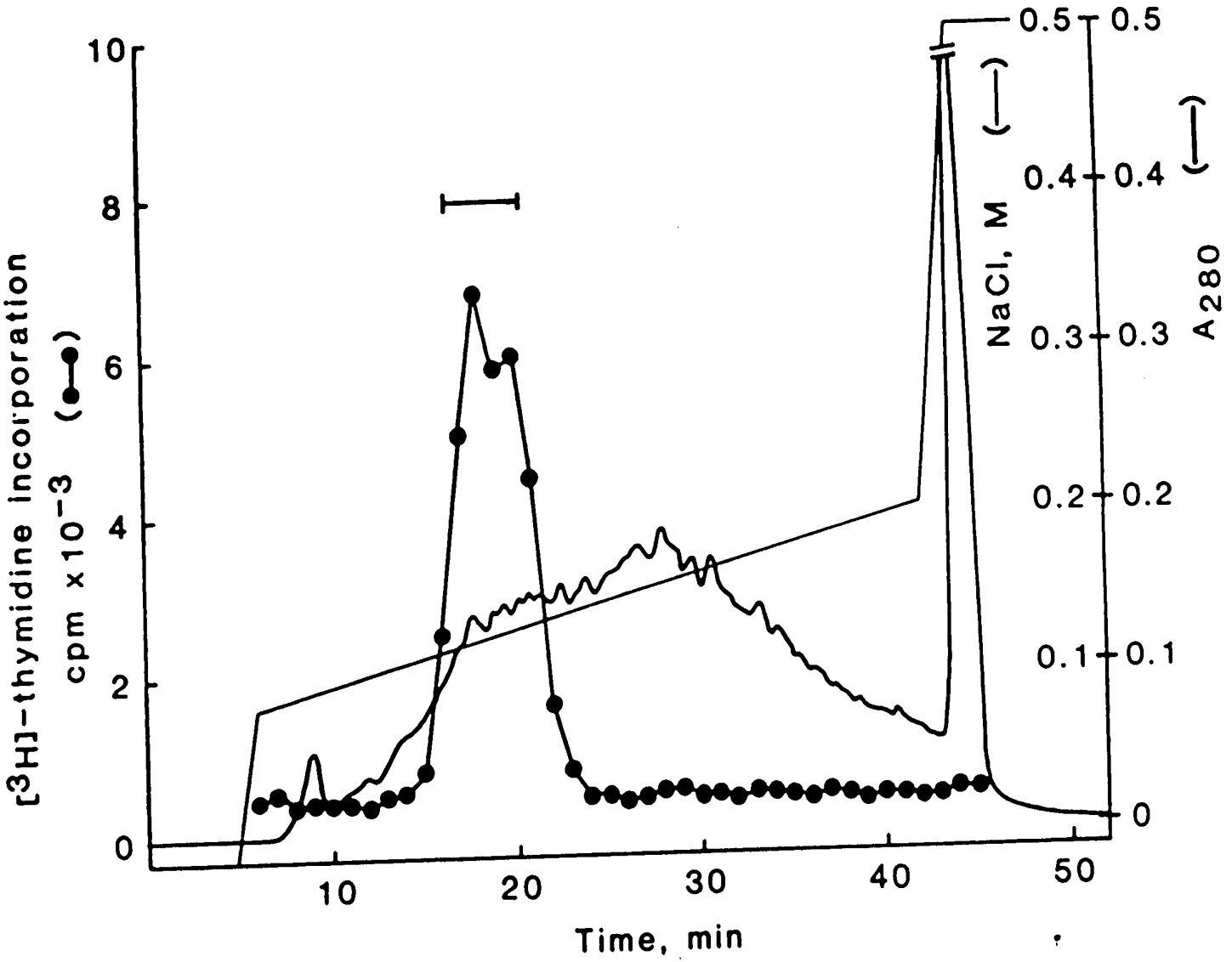

[3H]-thymidine incorporation
cpm x10-3
NaCl, M
A280
Time, min
0
2
4
6
8
10
0.1
0.2
0.3
0.4
0.5
10
20
30
40
50

FIG.3

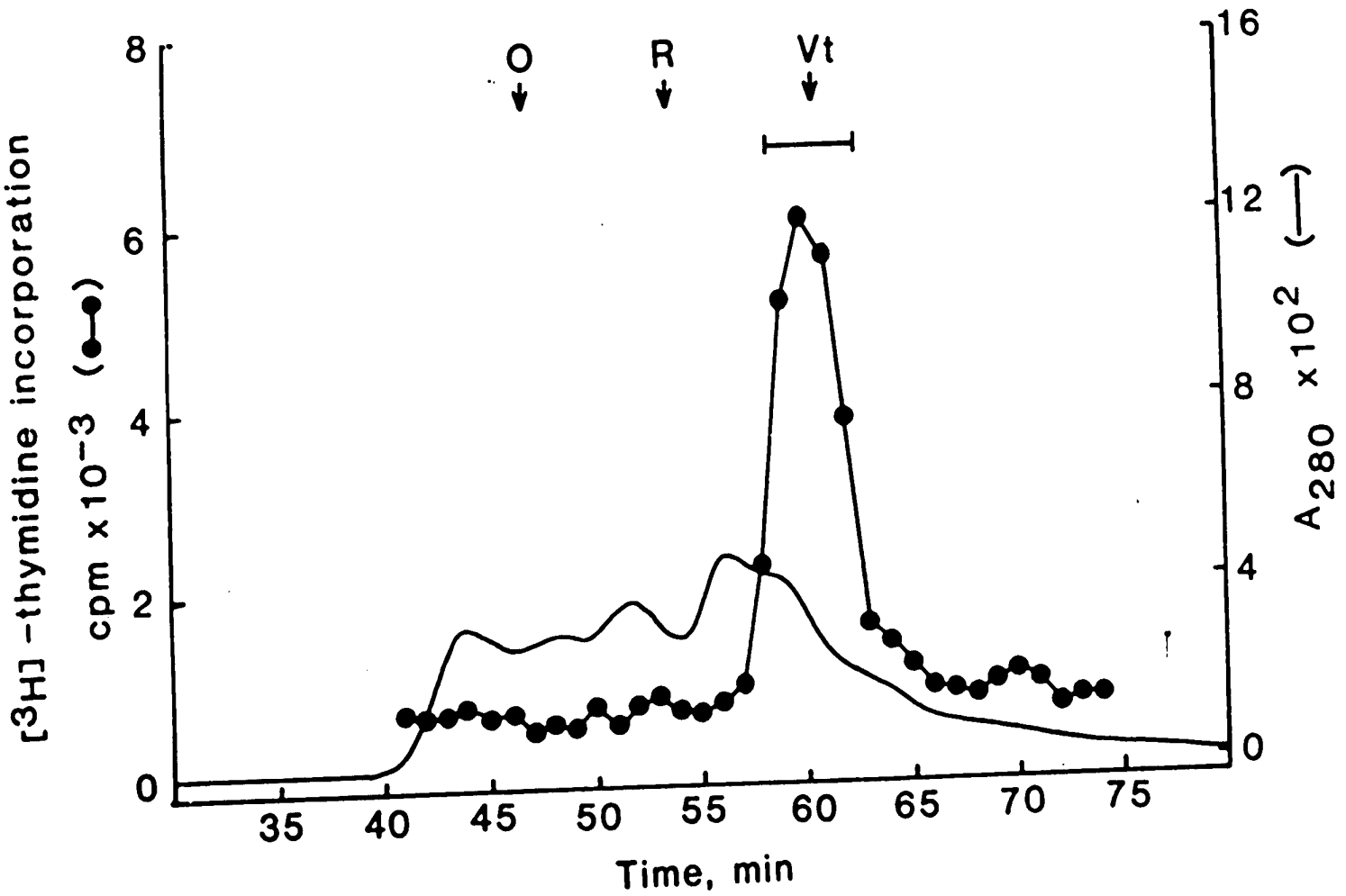

O
R
Vt
8
6
4
2
0
16
12
8
4
0
35
40
45
50
55
60
65
70
75
Time, min
[3H]-thymidine incorporation
cpm x10-3 (●—●)
A280 x10² (—)

FIG. 4

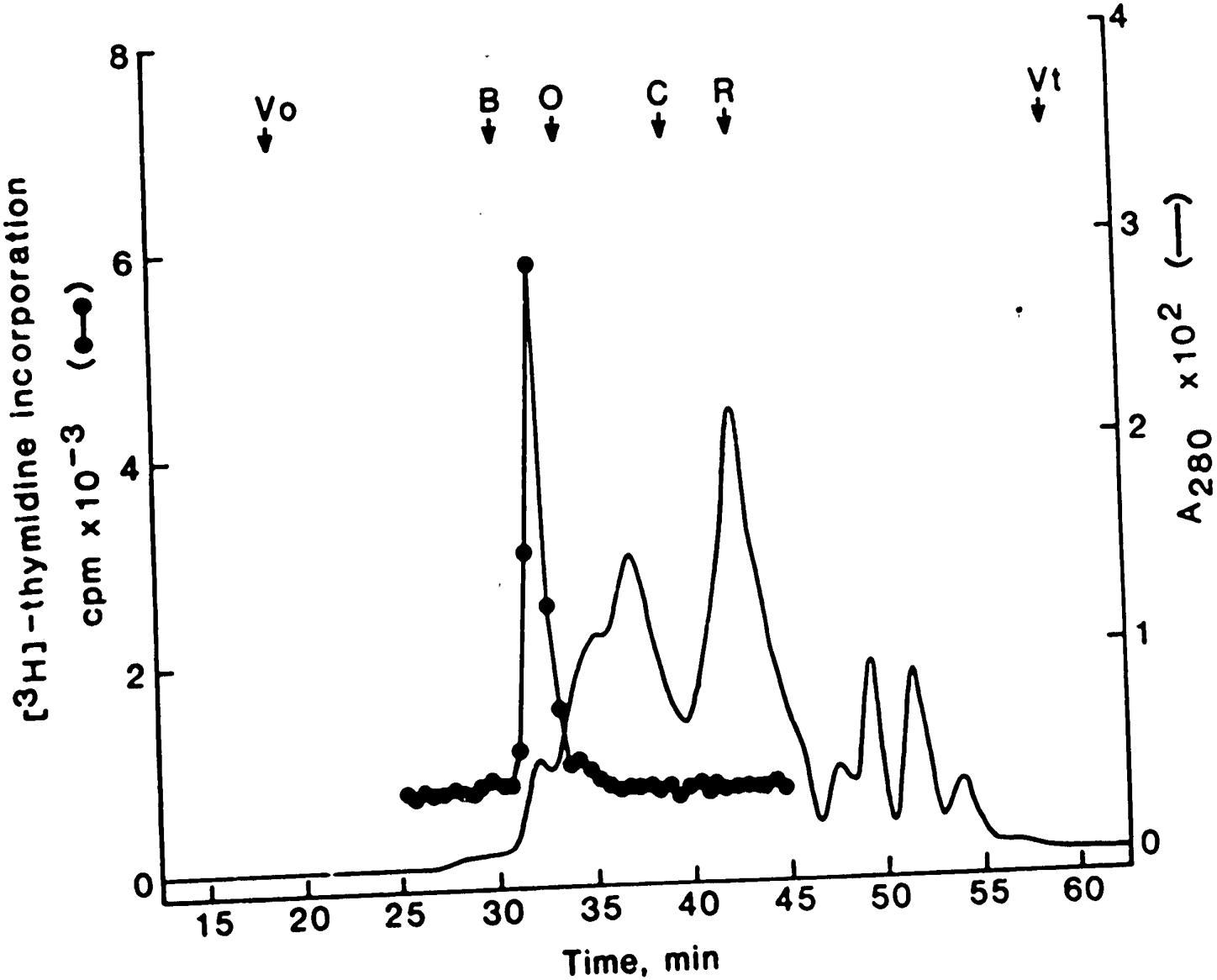

Vo
B
O
C
R
Vt
[3H]-thymidine incorporation
cpm x10-3 (●—●)
A280 x10^2 (—)
0
2
4
6
8
0
1
2
3
4
15
20
25
30
35
40
45
50
55
60
Time, min

FIG.5A

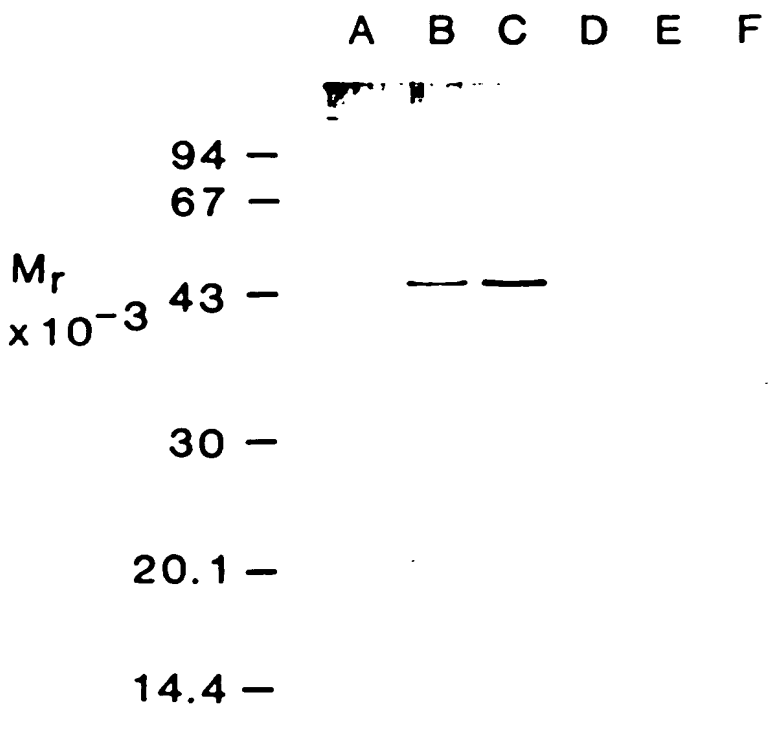
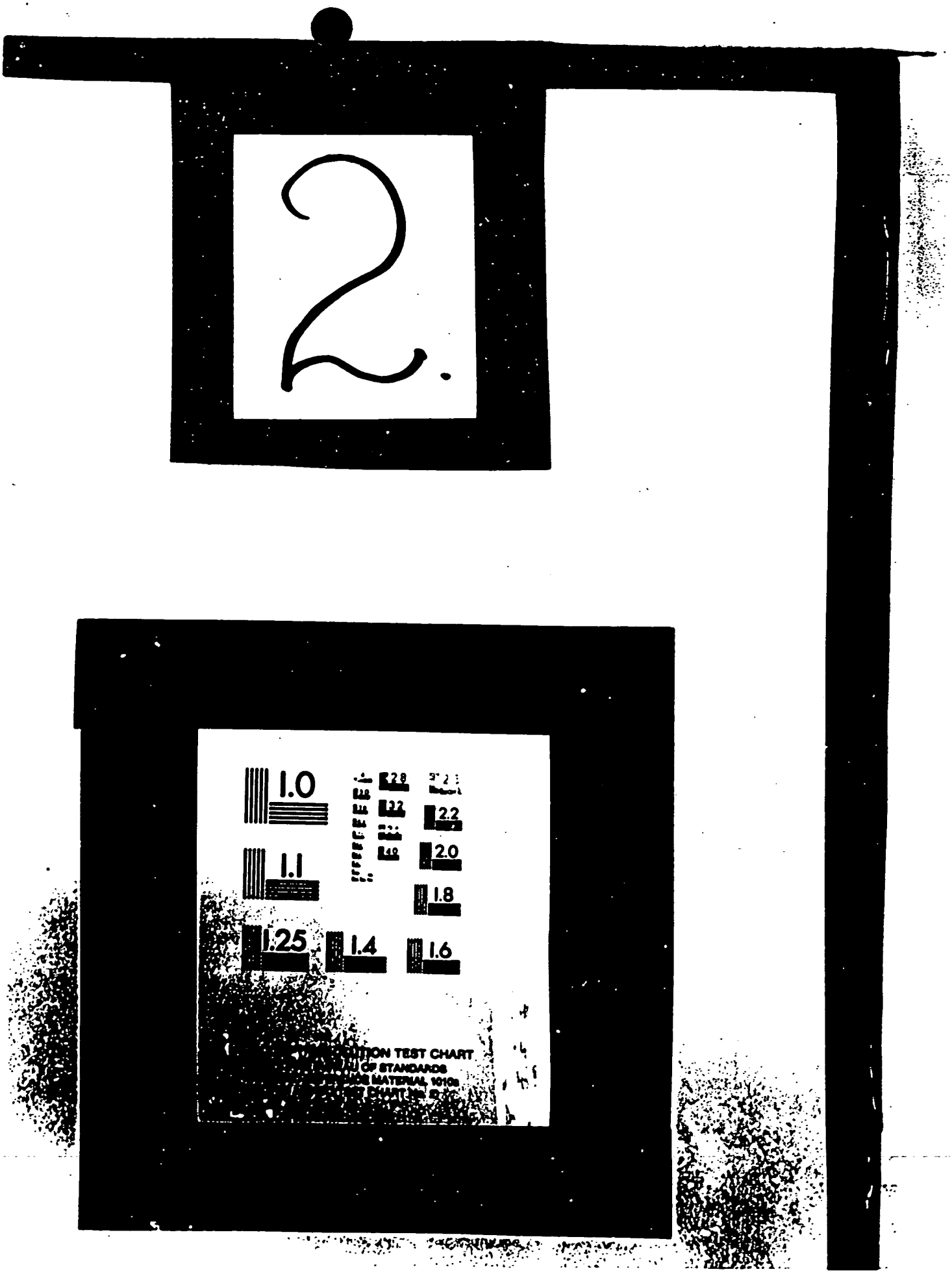


FIG.5B


2.
1.0
1.1
1.25
1.4
1.6
2.0
1.8
2.2

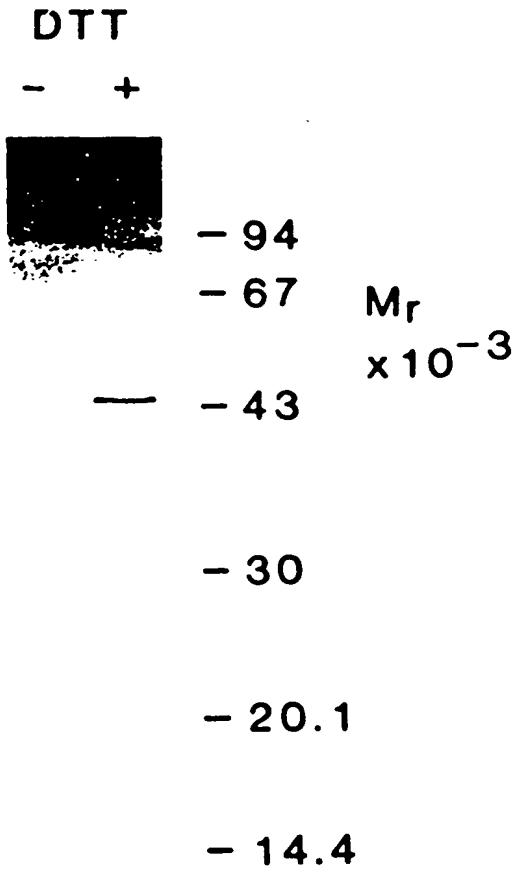


FIG.5C

FIG.6A

Hydroxylapatite chromatography of PD-ECGF

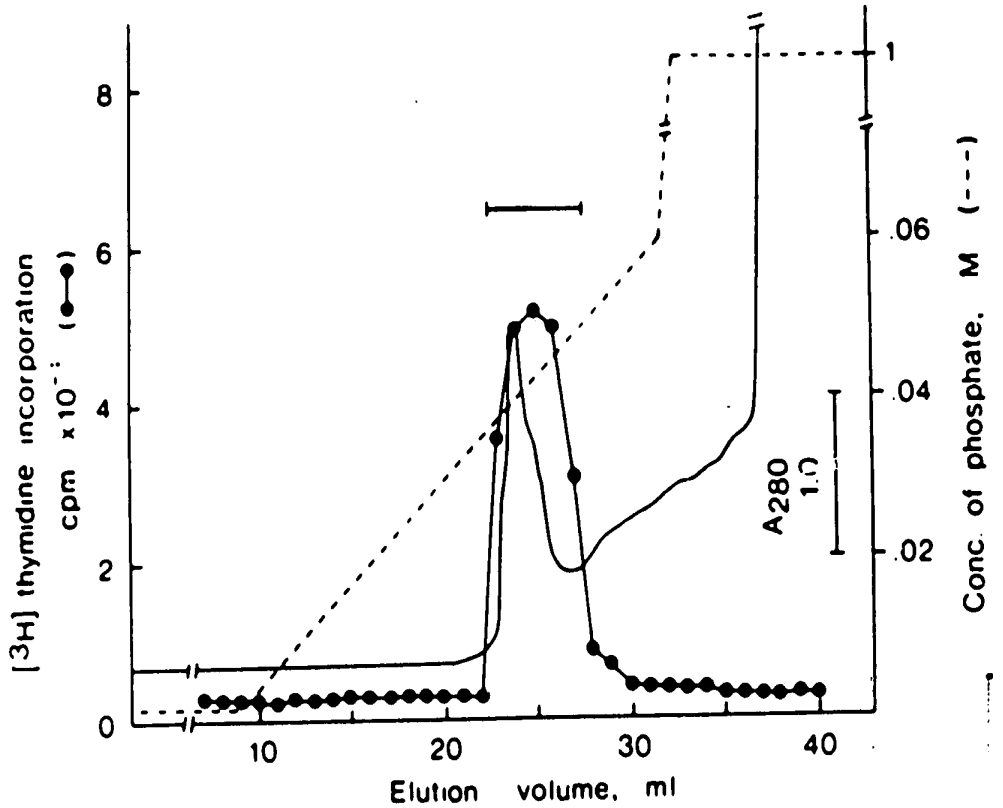


FIG.6B

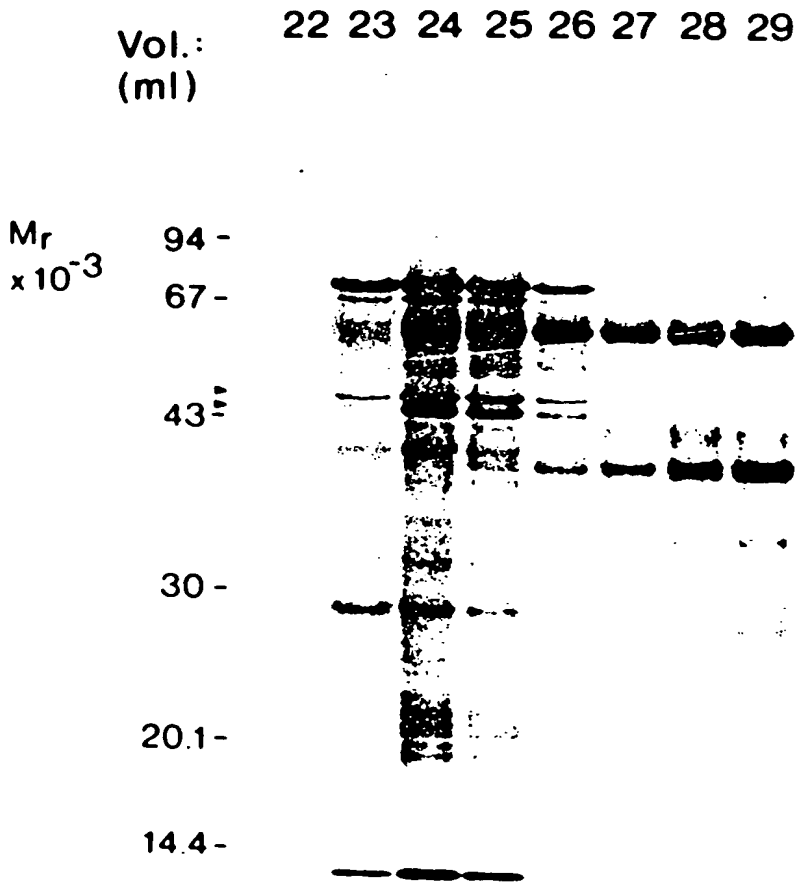


FIG.7A

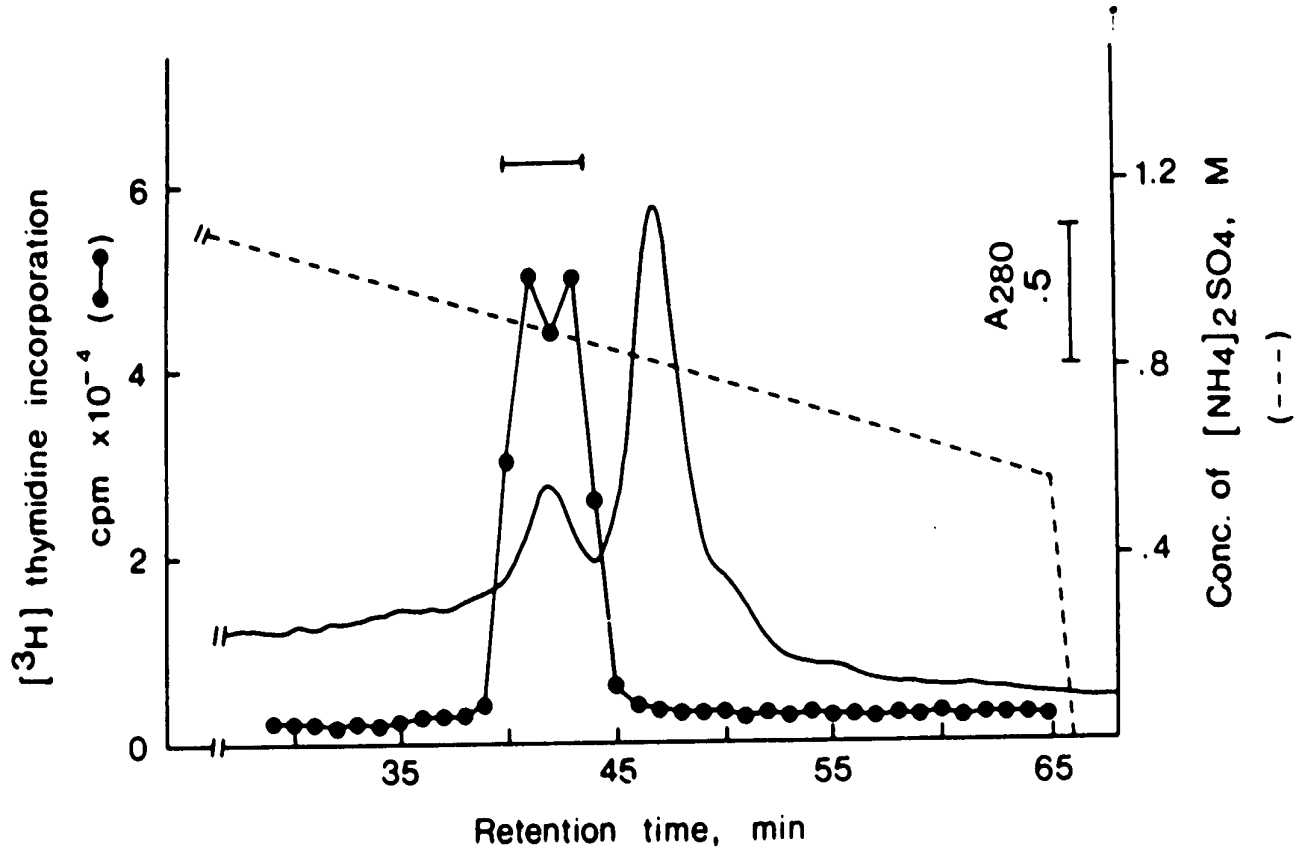

[3H] thymidine incorporation
cpm x10⁻⁴ (●—●)
0
2
4
6
35
45
55
65
Retention time, min
A280
.5
1.2
.8
.4
Conc. of [NH4]2SO4, M
(- - -)

FIG.7B

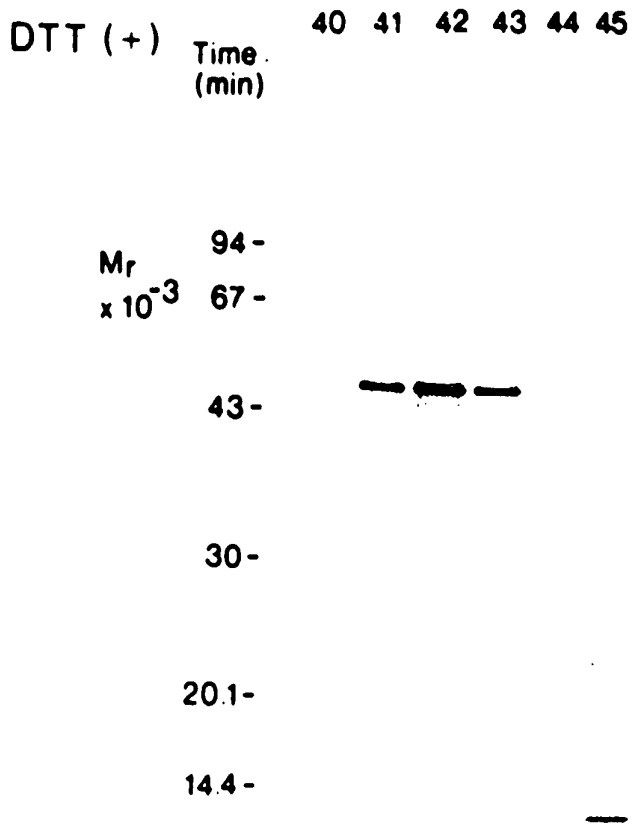
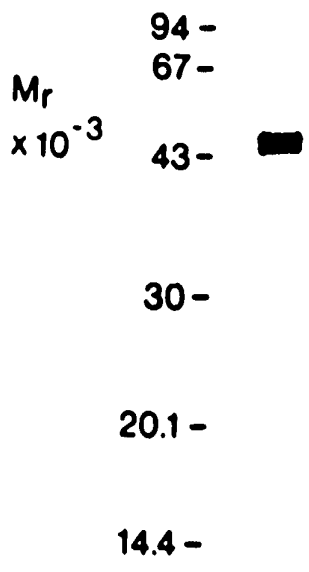

DTT (+)
Time (min)
40 41 42 43 44 45
Mr x 10-3
94-
67-
43-
30-
20.1-
14.4-

FIG.7C


DTT (-)
Mr x 10-3
94-
67-
43-
30-
20.1-
14.4-

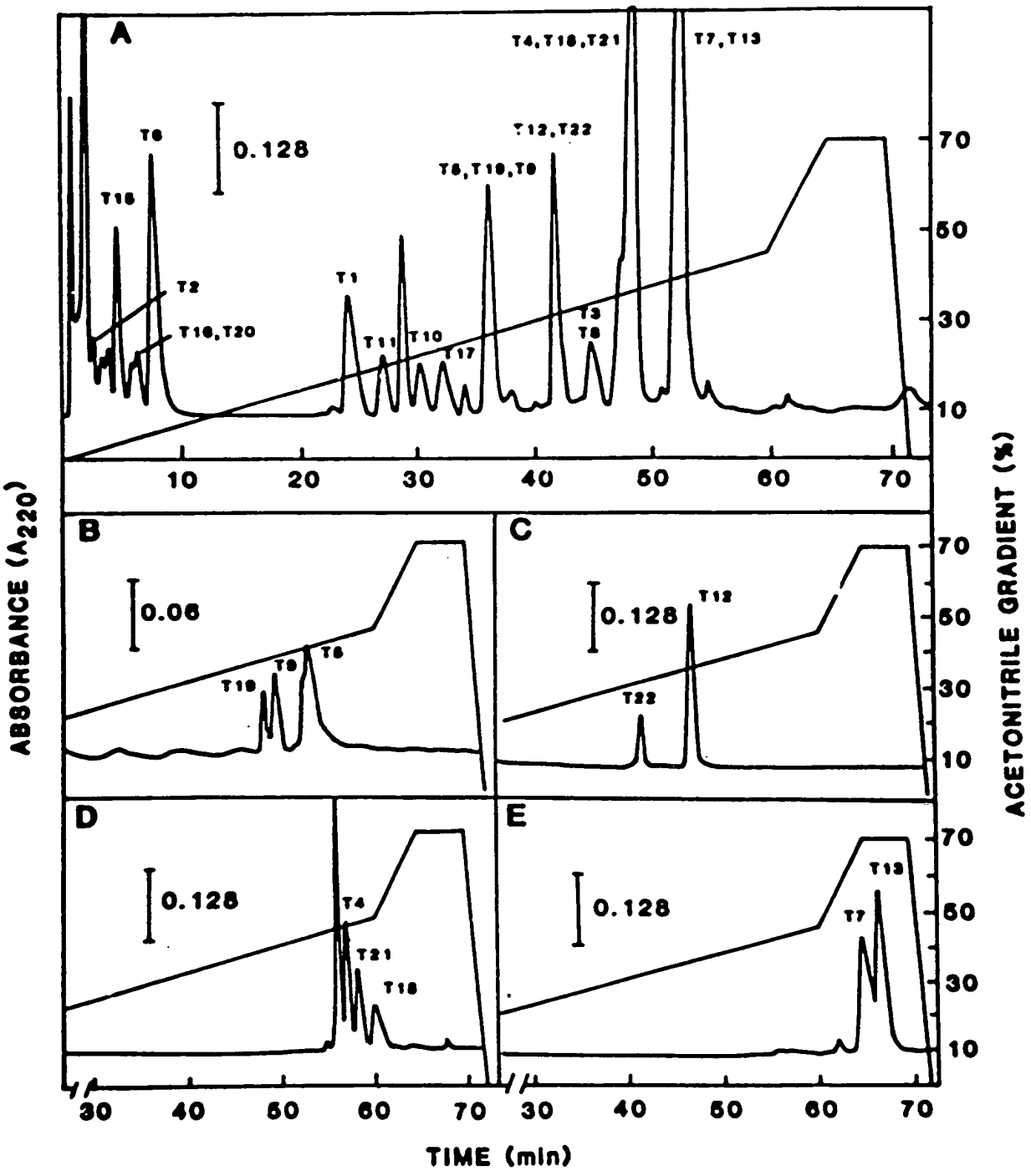

A
B
C
D
E
0.128
0.06
T6
T16
T2
T16,T20
T1
T11
T10
T17
T5,T19,T9
T12,T22
T4,T18,T21
T7,T13
T3
T8
T19
T9
T8
T12
T22
T4
T21
T18
T7
T13
10
20
30
40
50
60
70
ABSORBANCE (A220)
ACETONITRILE GRADIENT (%)
TIME (min)

FIG.8

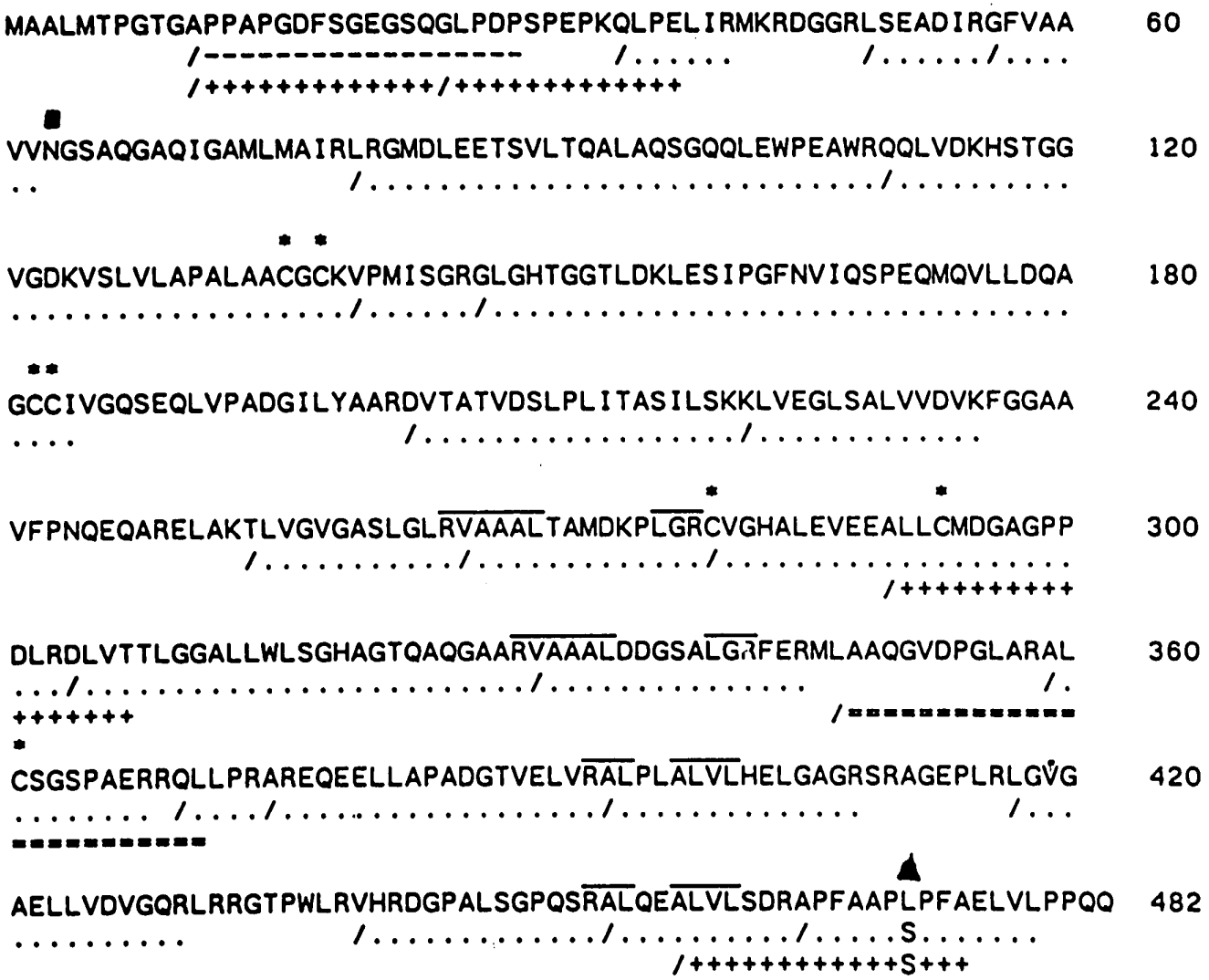

MAALMTPGTGAPPAPGDFSGEGSQGLPDPSPEPKQLPELIRMKRDGGRLSEADIRGFVAA 60
VVNGSAQGAQIGAMLMAIRLRGMDLEETSVLTQALAQSGQQLEWPEAWRQQLVDKHSTGG 120
VGDKVSLVLAPALAACGCKVPMISGRGLGHTGGTLDKLESIPGFNVIQSPEQMQVLLDQA 180
GCCIVGQSEQLVPADGILYAARDVTATVDSLPLITASILSKKLVEGLSALVVDVKFGGAA 240
VFPNQEQARELAKTLVGVGASLGLRVAAALTAMDKPLGRCVGHALEVEEALLCMDGAGPP 300
DLRDLVTTLGGALLWLSGHAGTQAQGAARVAAALDDGSALGRFERMLAAQGVDPGLARAL 360
CSGSPAERRQLLPRAREQEELLAPADGTVELVRALPLALVLHELGAGRSRAGEPLRLGVG 420
AELLVDVGQRLRRGTPWLRVHRDGPALSGPQSRALQEALVLSDRAPFAAPLPFAELVLPPQQ 482

FIG.9

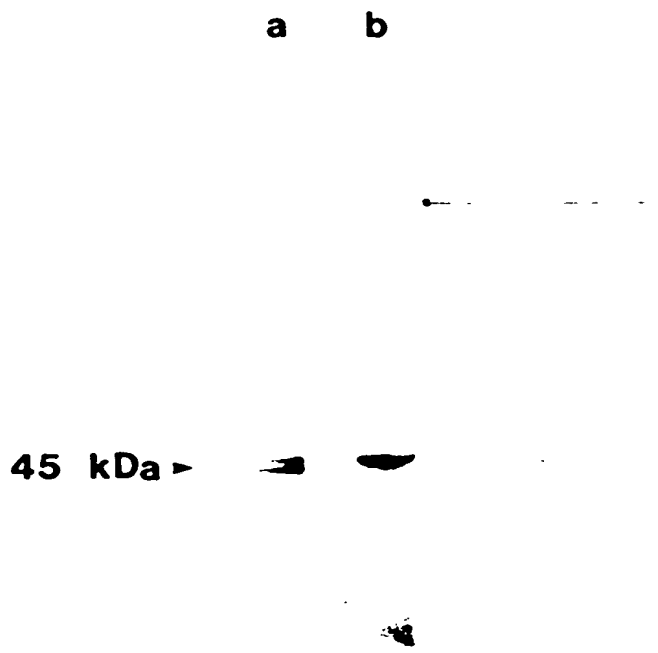


FIG.10

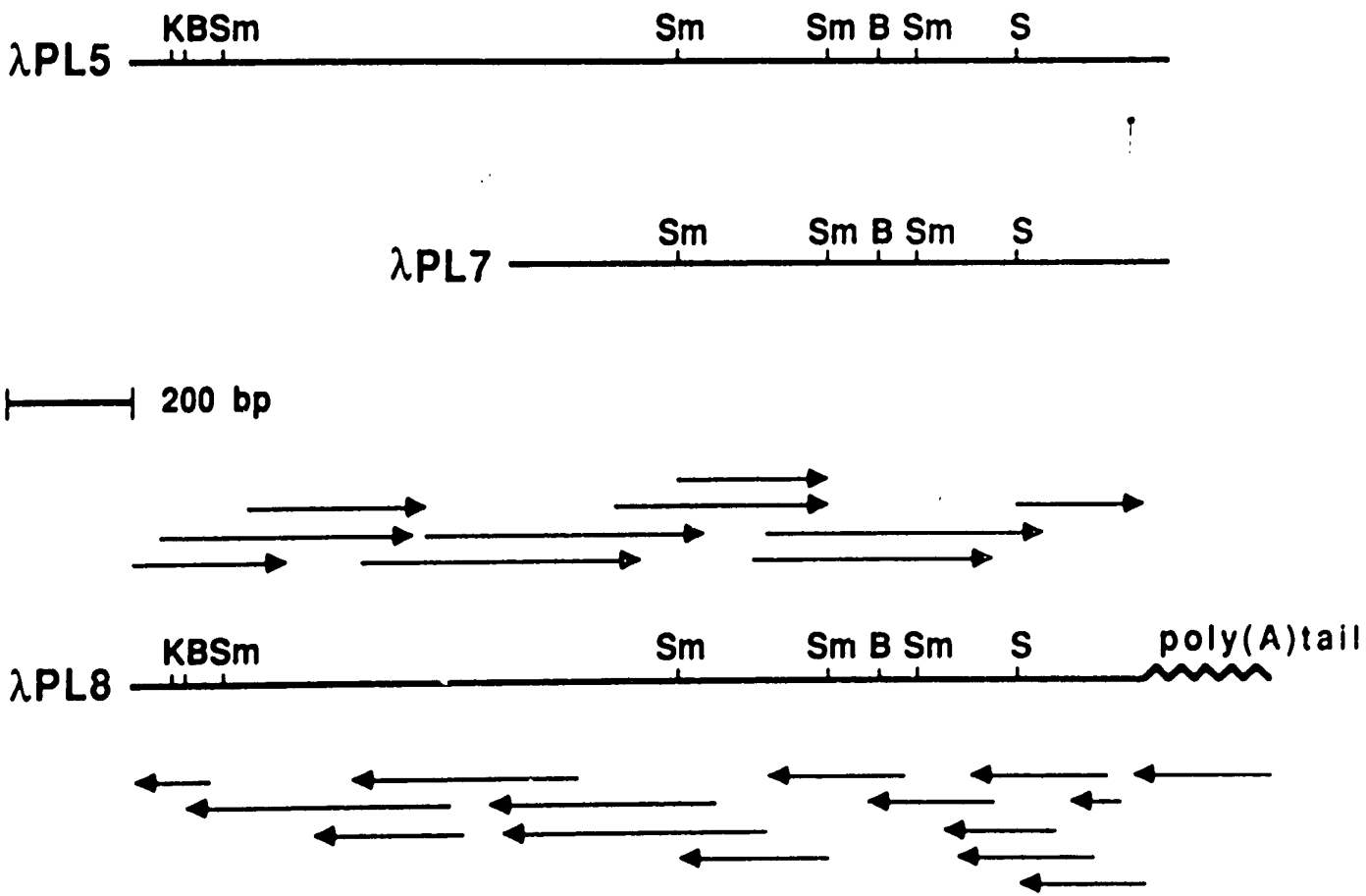

λPL5
KBSm
Sm
Sm B Sm
S
λPL7
Sm
Sm B Sm
S
200 bp
λPL8
KBSm
Sm
Sm B Sm
S
poly(A)tail

FIG.IIA

```
   1 GGGCAGTGGA   CCGCTGTGCG   CGAACCCTGA   ACCCTACGGT   CCCGACCCGC   GGGCGAGGCC
  61 GGGTACCTGG   GCTGGGATCC   GGAGCAAGCG   GGCGAGGGCA   GCGCCCTAAG   CAGGCCCGGA
                                                                  258
 121 GCG ATG GCA GCC TTG ATG ACC CCG GGA ACC GGG GCC CCA CCC GCG CCT GGT GAC TTC TCC
   1     Met Ala Ala Leu Met Thr Pro Gly Thr Gly Ala Pro Pro Ala Pro Gly Asp Phe Ser

 181 GGG GAA GGG AGC CAG GGA CTT CCC GAC CCT TCG CCA GAG CCC AAG CAG CTC CCG GAG CTG
  20 Gly Glu Gly Ser Gln Gly Leu Pro Asp Pro Ser Pro Glu Pro Lys Gln Leu Pro Glu Leu

 241 ATC CGC ATG AAG CGA GAC GGA GGC CGC CTG AGC GAA GCG GAC ATC AGG GGC TTC GTG GCC
  40 Ile Arg Met Lys Arg Asp Gly Gly Arg Leu Ser Glu Ala Asp Ile Arg Gly Phe Val Ala

 301 GCT GTG GTG AAT GGG AGC GCG CAG GGC GCA CAG ATC GGG GCC ATG CTG ATG GCC ATC CGA
  60 Ala Val Val Asn Gly Ser Ala Gln Gly Ala Gln Ile Gly Ala Met Leu Met Ala Ile Arg

 361 CTT CGG GGC ATG GAT CTG GAG GAG ACC TCG GTG CTG ACC CAG GCC CTG GCT CAG TCG GGA
  80 Leu Arg Gly Met Asp Leu Glu Glu Thr Ser Val Leu Thr Gln Ala Leu Ala Gln Ser Gly

 421 CAG CAG CTG GAG TGG CCA GAG GCC TGG CGC CAG CAG CTT GTG GAC AAG CAT TCC ACA GGC
 100 Gln Gln Leu Glu Trp Pro Glu Ala Trp Arg Gln Gln Leu Val Asp Lys His Ser Thr Gly

 481 GGT GTG GGT GAC AAG GTC AGC CTG GTC CTC GCA CCT GCC CTG GCG GCA TGT GGC TGC AAG
 120 Gly Val Gly Asp Lys Val Ser Leu Val Leu Ala Pro Ala Leu Ala Ala Cys Gly Cys Lys
                                                 268
 541 GTG CCA ATG ATC AGC GGA CGT GGT CTG GGG CAC ACA GGA GGC ACC TTG GAT AAG CTG GAG
 140 Val Pro Met Ile Ser Gly Arg Gly Leu Gly His Thr Gly Gly Thr Leu Asp Lys Leu Glu
```

FIG.11B

601 TCT ATT CCT GGA TTC AAT GTC ATC CAG AGC CCA GAG CAG ATG CAA GTG CTG CTG GAC CAG
160 S r Il Pro Gly Phe Asn Val Il Gln Ser Pro Glu Gln M t Gln Val Leu L u Asp Gln

661 GCG GGC TGC TGT ATC GTG GGT CAG AGT GAG CAG CTG GTT CCT GCG GAC GGA ATC CTA TAT
180 Ala Gly Cys Cys Ile Val Gly Gln Ser Glu Gln Leu Val Pro Ala Asp Gly Ile Leu Tyr

721 GCA GCC AGA GAT GTG ACA GCC ACC GTG GAC AGC CTG CCA CTC ATC ACA GCC TCC ATT CTC
200 Ala Ala Arg Asp Val Thr Ala Thr Val Asp Ser Leu Pro Leu Ile Thr Ala Ser Ile Leu

781 AGT AAG AAA CTC GTG GAG GGG CTG TCC GCT CTG GTG GTG GAC GTT AAG TTC GGA GGG GCC
220 Ser Lys Lys Leu Val Glu Gly Leu Ser Ala Leu Val Val Asp Val Lys Phe Gly Gly Ala

841 GCC GTC TTC CCC AAC CAG GAG CAG GCC CGG GAG CTG GCA AAG ACG CTG GTT GGC GTG GGA
240 Ala Val Phe Pro Asn Gln Glu Gln Ala Arg Glu Leu Ala Lys Thr Leu Val Gly Val Gly
231
901 GCC AGC CTA GGG CTT CGG GTC GCG GCA GGG CTG ACC GGC ATG GAC AAG CCC CTG GGT CGC
260 Ala Ser Leu Gly Leu Arg Val Ala Ala Ala Leu Thr Ala Met Asp Lys Pro Leu Gly Arg
278
961 TGC GTG GGC CAC GCC CTG GAG GTG GAG GAG GCG CTG CTC TGC ATG GAC GGC GCA GGC CCG
280 Cys Val Gly His Ala Leu Glu Val Glu Glu Ala Leu Leu Cys Met Asp Gly Ala Gly Pro

1021 CCA GAC TTA AGG GAC CTG GTC ACC ACG CTC GGG GGC GCC CTG CTC TGG CTC AGC GGA CAC
300 Pro Asp Leu Arg Asp Leu Val Thr Thr Leu Gly Gly Ala Leu Leu Trp Leu Ser Gly His

1081 GCG GGG ACT CAG GCC CAG GGC GCT GCC CGG GTG GCC GCG GCG CTG GAC GAC GGC TCG GCC
320 Ala Gly Thr Gln Ala Gln Gly Ala Ala Arg Val Ala Ala Ala Leu Asp Asp Gly Ser Ala
259
1141 CTT GGC CGC TTC GAG CGG ATG CTG GCG GCG CAG GGC GTG GAT CCC GGT CTG GCC CGA GCC
340 Leu Gly Arg Phe Glu Arg Met Leu Ala Ala Gln Gly Val Asp Pro Gly Leu Ala Arg Ala

FIG.11B CONT.'

1201 CTG TGC TCG GGA AGT CCC GCA GAA CGC CGG CAG CTG CTG CCT CGC GCC CGG GAG CAG GAG
360 Leu Cys Ser Gly Ser Pro Ala Glu Arg Arg Gln Leu Leu Pro Arg Ala Arg Glu Gln Glu

1261 GAG CTG CTG GCG CCC GCA GAT GGC ACC GTG GAG CTG GTC CGG GCG CTG CCG CTG GCG CTC
380 Glu Leu Leu Ala Pro Ala Asp Gly Thr Val Glu Leu Val Arg Ala Leu Pro Leu Ala Leu

1321 GTG CTG CAC GAG CTC GGG GCC GGC CGC AGC CGC GCT GGG GAG CCG CTC CGC CTG GGG GTG
400 Val Leu His Glu Leu Gly Ala Gly Arg Ser Arg Ala Gly Glu Pro Leu Arg Leu Gly Val

1381 GGC GCA GAG CTG CTG GTC GAC GTG GGT CAG AGG CTG CGC CGT GGG ACC CCC TGG CTC CGC
420 Gly Ala Glu Leu Leu Val Asp Val Gly Gln Arg Leu Arg Arg Gly Thr Pro Trp Leu Arg

1441 GTG CAC CGG GAC GGC CCC GCG CTC AGC GGC CCG CAG AGC CGC GCC CTG CAG GAG GCG CTC
440 Val His Arg Asp Gly Pro Ala Leu Ser Gly Pro Gln Ser Arg Ala Leu Gln Glu Ala Leu

1501 GTA CTC TCC GAC CGC GCG CCA TTC GCC GCC CCC TTG CCC TTC GCA GAG CTC GTT CTG CCG
460 Val Leu Ser Asp Arg Ala Pro Phe Ala Ala Pro Leu Pro Phe Ala Glu Leu Val Leu Pro

1561 CCG CAG CAA TAA AGC TCC TTT GCC GCG AAA $(A)_n$
480 Pro Gln Gln •

FIG.11B CONT.

Southern blot analysis of human PD-ECGF gene

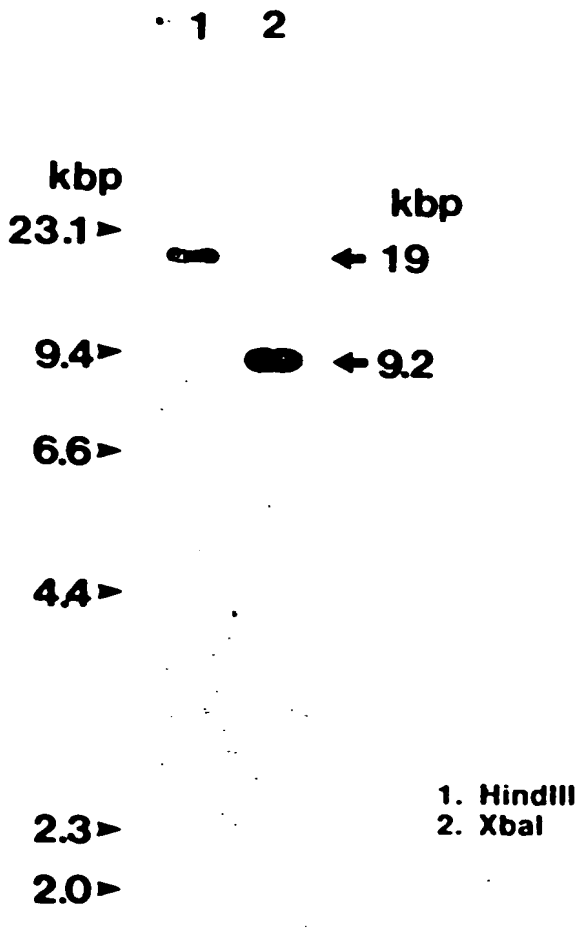


FIG.12A

Expression of PD-ECGF in human placenta

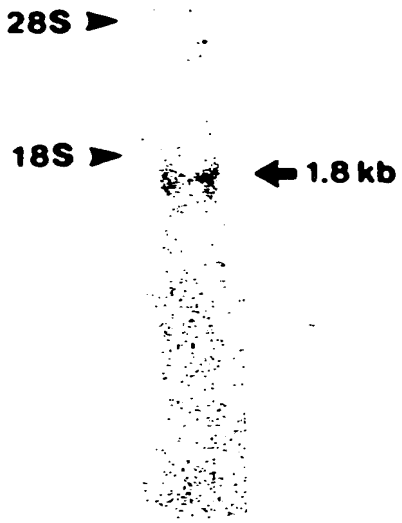
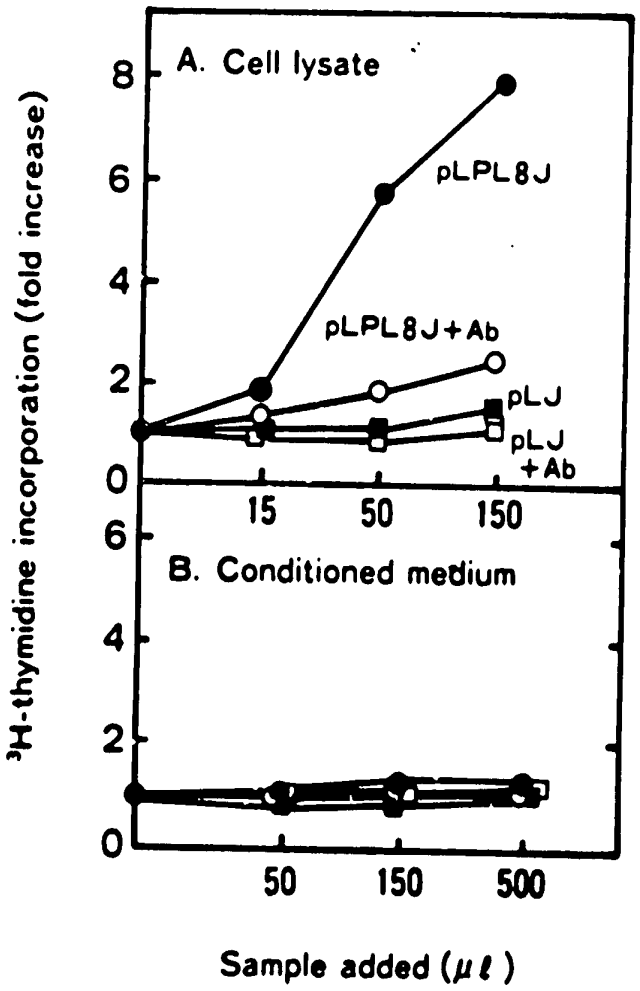


FIG.12B

FIG.13

Biosynthesis of PD-ECGF in NIH 3T3 Cells Transfected with pPL8



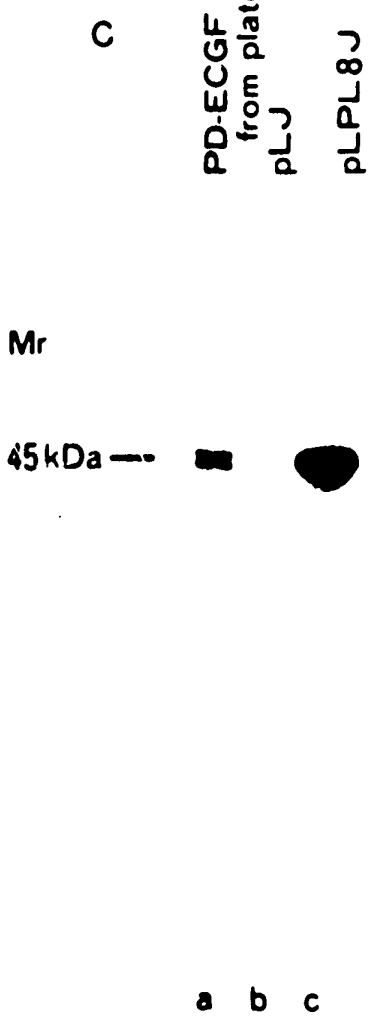
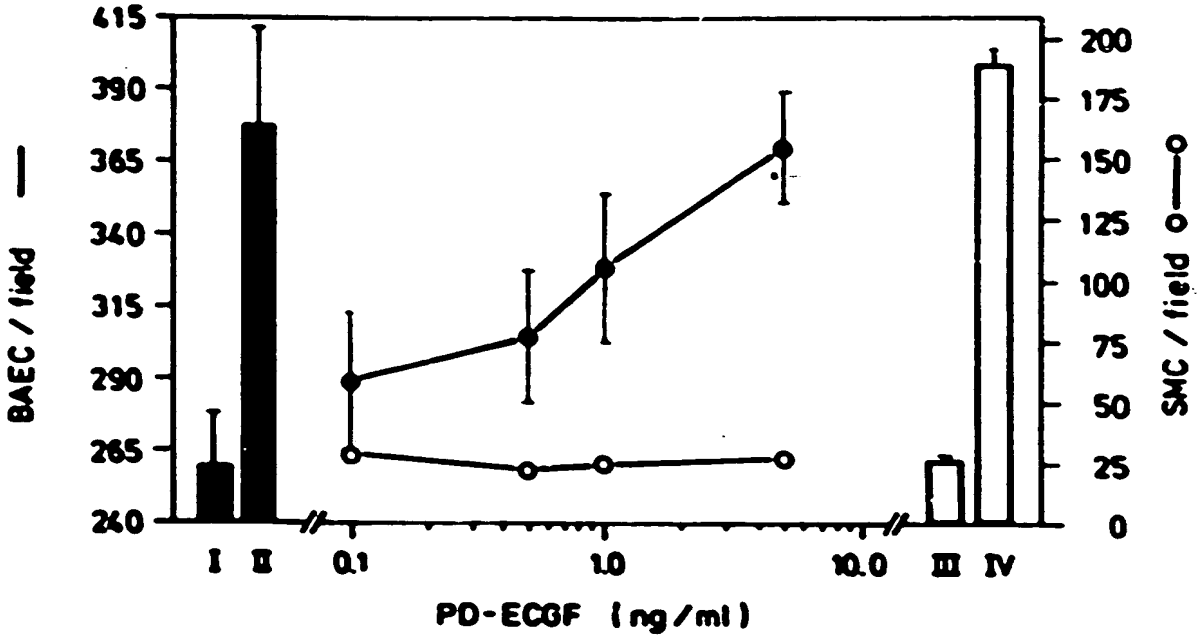


FIG.14A



Protein concentration of pure PD-ECGF in upper chamber (ng/ml)

Protein concentration of pure PD-ECGF in lower chamber (ng/ml)

	0	0.5	1	5
0	100	79	91	51
0.5	102	83	101	80
1	138	98	105	82
5	154	140	88	83

FIG.14B

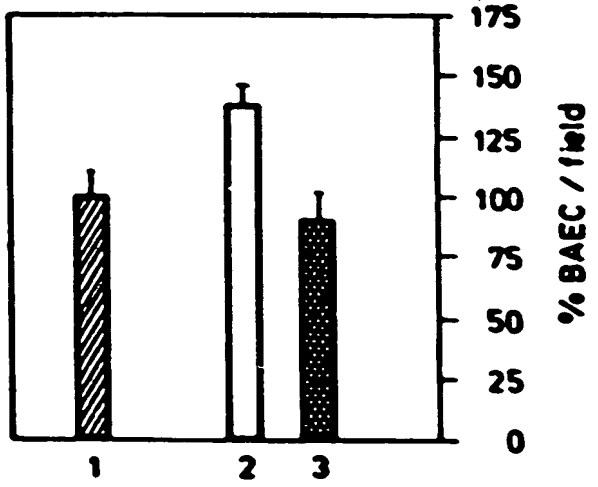


FIG.14C

FIG.15A

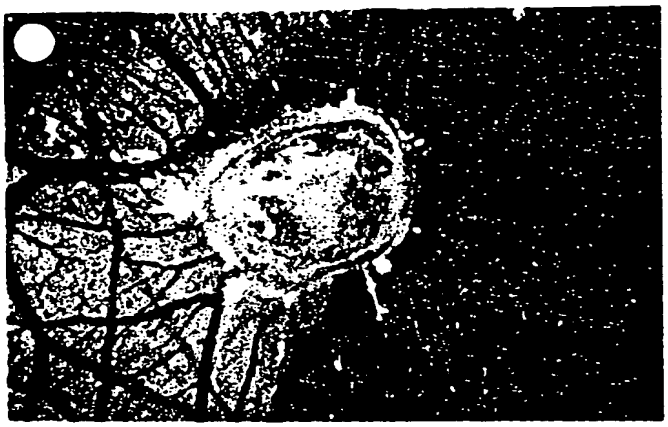


FIG.15B

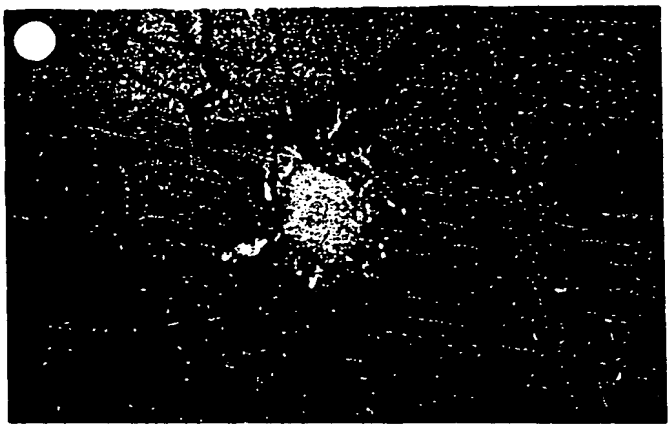


FIG.15C

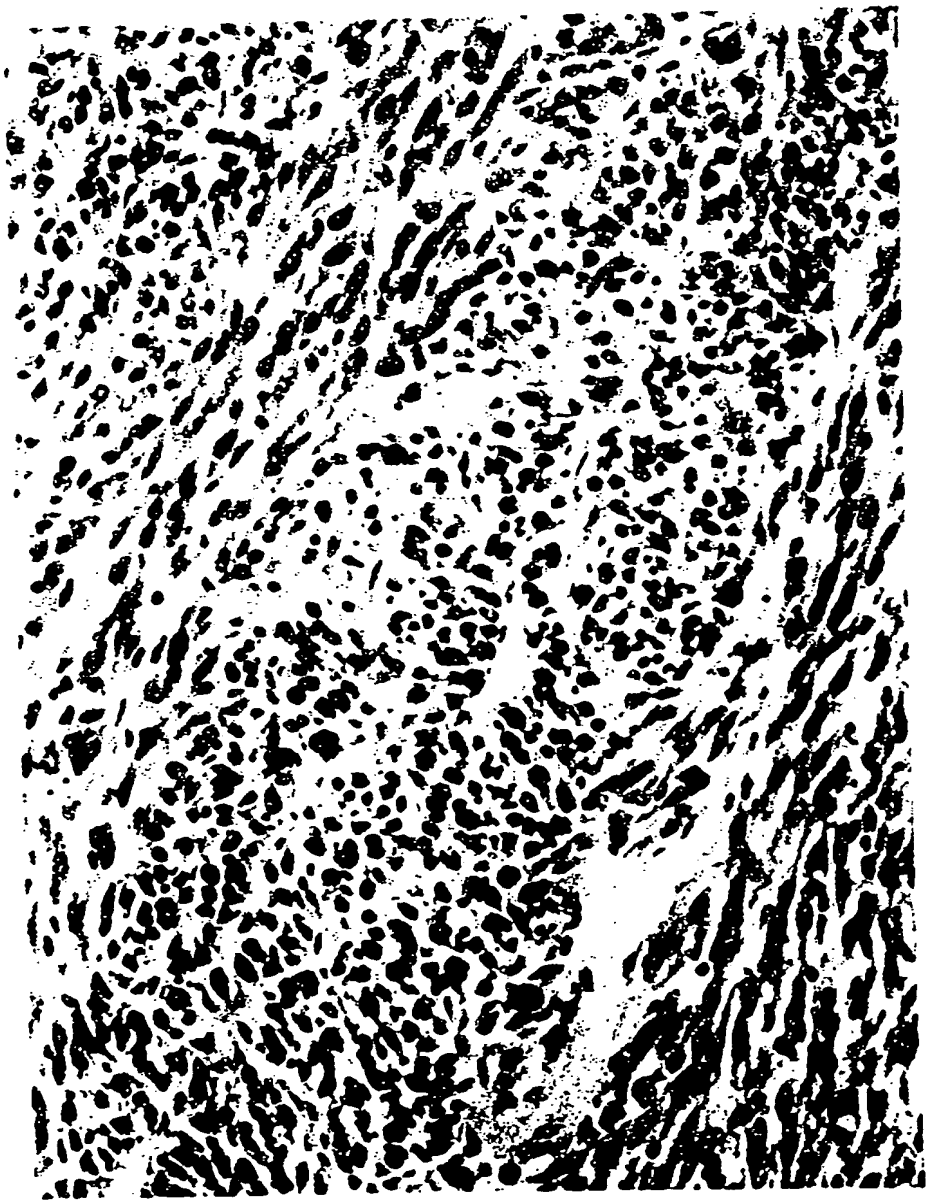


FIG.16A

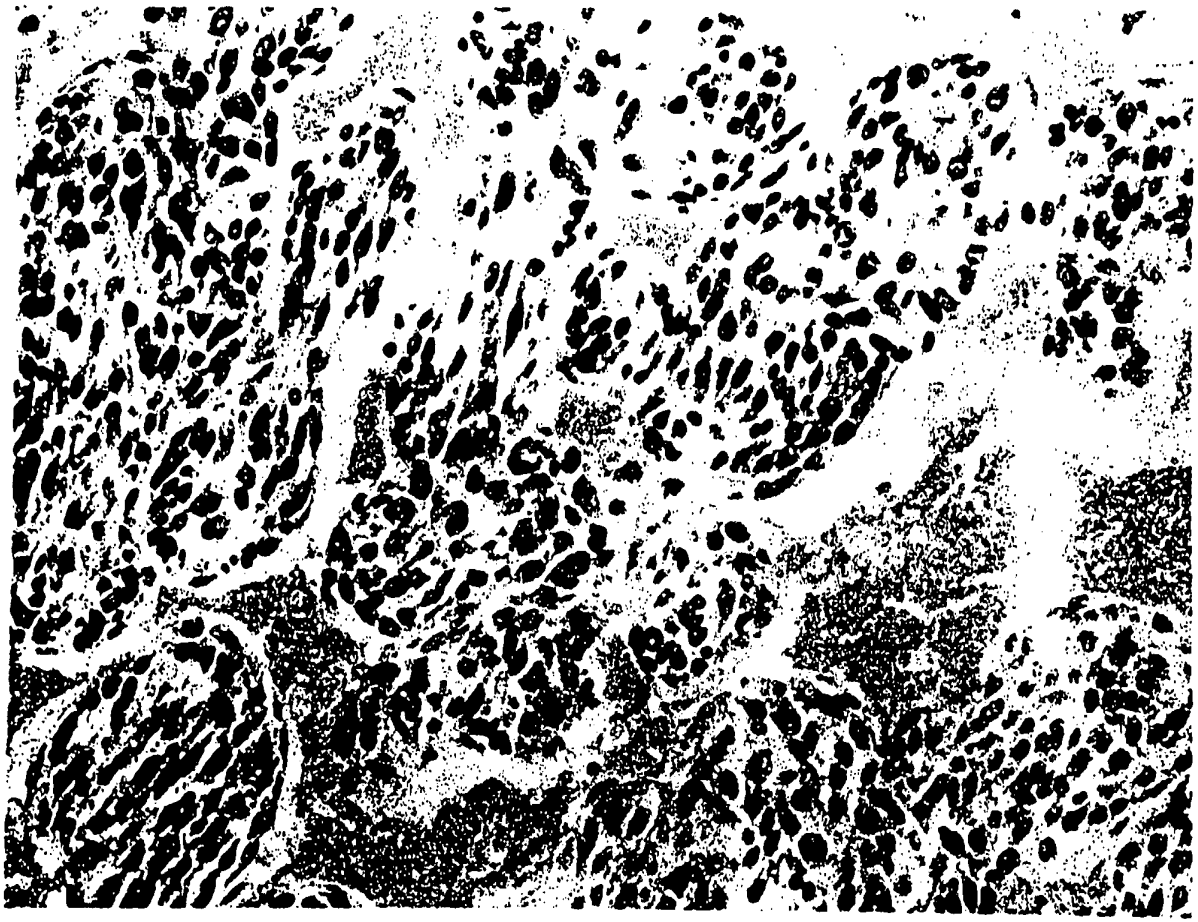
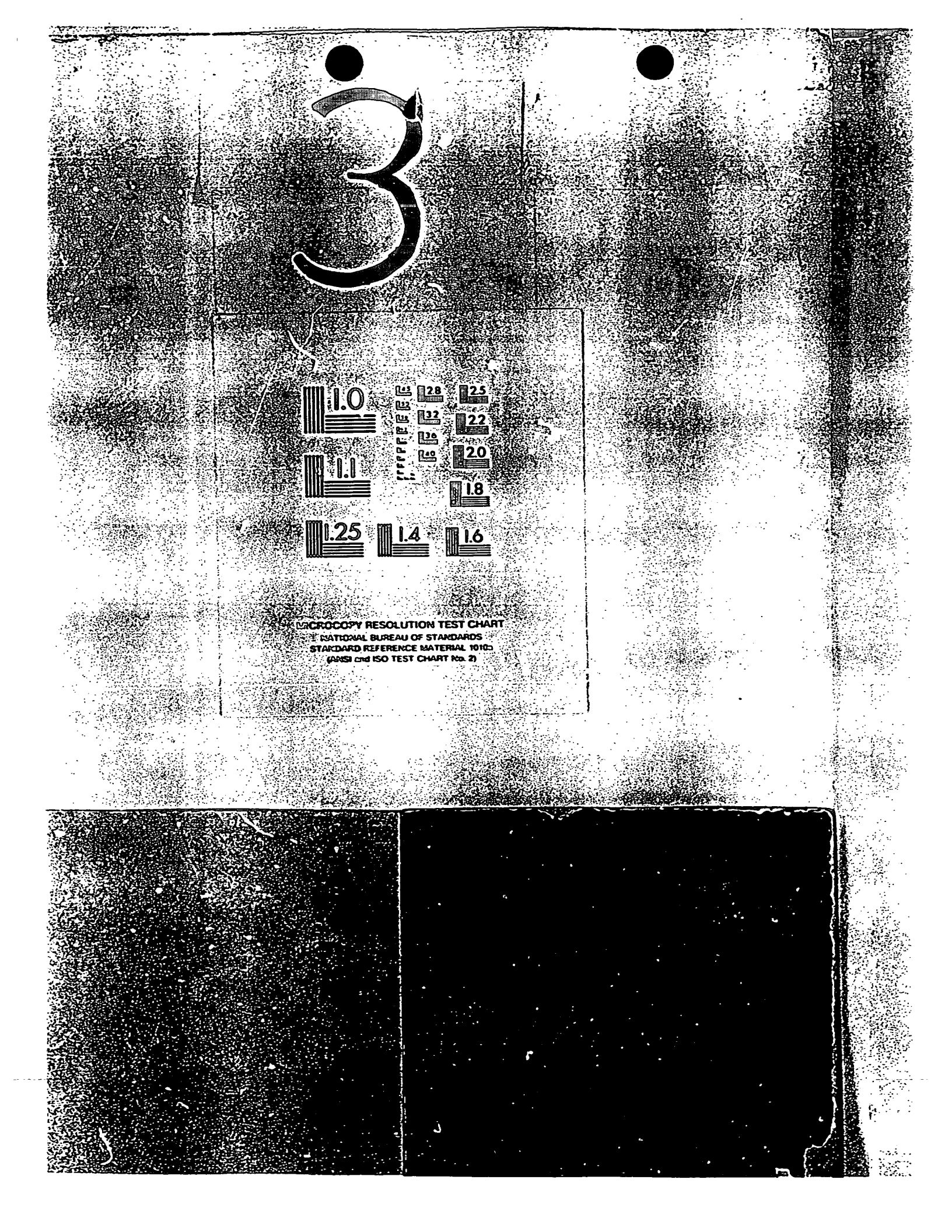


FIG.16B


3
1.0
1.1
1.25
1.4
1.6
2.8
2.5
3.2
2.2
3.6
2.0
4.0
1.8
MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)